Vol. XLIII No. 3

March 1954

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

The Kinetics of Degradation of Chloramphenicol in Solution. II. Over-all Disappearance Rate from Buffered Solutions. 129
Takeru Higuchi, Arnold D. Marcus, and *Charles D. Bias*

Comparison of a Microbiological and a Chromatographic Assay Method for Chloramphenicol. 135
Takeru Higuchi, Arnold D. Marcus, and *Charles D. Bias*

Antibacterial Activity of *Cassia reticulata* Willd. 139
Heber W. Youngken and *Robert A. Walsh*

Malabar Rauwolfia, *Rauwolfia micrantha* Hook. f. 141
Heber W. Youngken

A Preliminary Chemical Investigation of *Rauwolfia serpentina*. 144
Wendell L. Holt and *Christopher H. Costello*

Bifunctional Theophylline Derivatives and Corresponding Imidazolines. 148
George P. Hager and *Carl Kaiser*

Theophylline Derivatives. II. 8-Aralkyltheophyllines and Related Compounds. 152
George P. Hager, John C. Krantz, Jr., John B. Harmon, and *Raymond M. Burgison*

Theophylline Derivatives. III. 8-(9-Fluorenyl)-Theophylline and Related Compounds. 156
George P. Hager, Casimir T. Ichniowski, and *Bernard Misek*

Cyanocobalamin (Vitamin B_{12}). I. A Study of the Stability of Cyanocobalamin and Ascorbic Acid in Liquid Formulations. 159
Andrew Bartilucci and *Noel E. Foss*

The Synthesis of Possible Degradation Products of Nicotine. 163
Raymond N. Castle and *Alfred Burger*

Cardiovascular Actions of Some Simple Nucleic Acid Derivatives. 166
Virginia Sydow and *Raymond P. Ahlquist*

An Improved *in vivo* Method for Comparing the Constipative Effects of Drugs in Rabbits. 170
Wm. C. Van Arsdel, III, and *Norman A. David*

Extraction and Periodate Cleavage Studies of the Tannin Obtained from *Pinus caribaea*, Morelet. 172
A. I. Dines and *F. W. Bope*

Protective Effect of Liver Residue on Immature Male Rats Fed Toxic Doses of Acetylsalicylic Acid. 175
B. H. Ershoff, H. B. McWilliams, and *E. W. Thurston*

Stability of Some Bactericidal Chlorine-Liberating Compounds in Special Ointment Bases. 178
Abraham Taub, Fanchon Hart, and *Stanley Kassimir*

The Preparation and Evaluation of Tangerine Peel Tincture and Tangerine Syrup. 181
William J. Sheffield and *Herman O. Thompson*

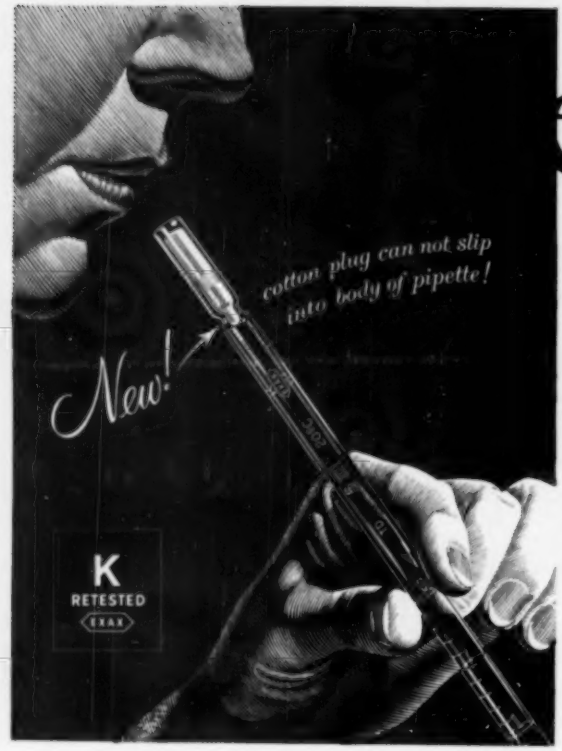
Comparative Studies on the Toxicity of Washed Cells of *Bacillus megaterium* and Brewers' Yeast. 185
Anthony M. Ambrose and *Floyd DeEds*

A Study of the Root System of *Hemerocallis fulva* L. I. Morphology and Histology. 188
Edward P. Claus

Book Notices. 191

Advertising. Cover pages 2, 3, 4, and pages I–VIII

Copyright 1954, by the American Pharmaceutical Association



ew!

KIMBLE PIPETTES with cotton plug constriction

They are legible—All markings are clearly indicated with Kimble permanent, *fused-in* filler.

They are retested—Each pipette is tested during manufacture, then individually retested for accuracy before shipment.

These new Kimble Pipettes combine Kimble quality with new efficiencies. Their new design prevents the cotton plug from slipping down into the main body.

The constriction is large enough for easy cleaning and in no way weakens the tube; nor does it alter flow characteristics.

Your Kimble laboratory supply dealer will show you samples and supply complete information. Call or write him today.

KIMBLE LABORATORY GLASSWARE
AN (O) PRODUCT

OWENS-ILLINOIS
GENERAL OFFICES · TOLEDO 1, OHIO

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

SCIENTIFIC EDITION

Committee on Publications

Don E. Francke *Chairman*
Walter M. Chase
Robert P. Fischelis
F. Royce Franzoni
Hugo H. Schaefer

Editorial Advisory Board

John E. Christian
Walter C. Gakenheimer
George P. Hager
L. W. Hazleton
Elmer B. Vliet
H. W. Youngken, Jr.

JUSTIN L. POWERS, EDITOR
HELEN O. NICOL, EDITORIAL ASSISTANT

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5" x 11" in size and with 1" to 1.5" margins. In addition to the original typewritten copy, one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 200 words which includes a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Finally there should be a brief summary of conclusions and recommendations. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should

The Journal of the American Pharmaceutical Association is published monthly by the American Pharmaceutical Association in two editions: Scientific Edition and Practical Pharmacy Edition, under the supervision of the Committee on Publications of the Council of the Association. Publication office: 20th and Northampton Streets, Easton, Pennsylvania.

Editorial and Executive Offices—2215 Constitution Avenue, N. W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

Annual Subscription—Journal of the American Pharmaceutical Association, Scientific Edition and Practical Pharmacy Edition combined—United States and Pan America, $7.00; Canada, $7.70; other foreign, $8.00.

Journal of the American Pharmaceutical Association, Scientific Edition alone: United States and Pan America, $4.00; Canada, $4.35; other foreign, $4.50.

Journal of the American Pharmaceutical Association, Practical Pharmacy Edition alone: United States and Pan America, $4.00; Canada, $4.35; other foreign, $4.50.

Members of the American Pharmaceutical Association receive the combined subscription as a part of their annual dues. Members may request omission of either edition, but this will not reduce the amount of the annual dues.

Single copies—Journal of the American Pharmaceutical Association, Scientific Edition or Practical Pharmacy Edition: United States and Pan America, $0.35; Canada, $0.40; other foreign, $0.50.

Subscription rates are subject to change without notice.

Change of address—Members and subscribers should bear in mind that second-class matter is not forwarded by the Post Office, and should supply both the Post Office and the American Pharmaceutical Association with any change of address immediately upon its occurrence.

Claims for missing numbers will not be allowed if received more than 60 days after date of issue, or if loss was due to failure to give 30 days' notice of change of address. Claims asserting that Journals are "missing from files" cannot be allowed.

Entered as second class matter January 23, 1917, at the Post Office at Easton, Pennsylvania, under the Act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the 5th; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

References to books should be in the order given, (1) author's surname and initials, (2) title of the book, (3) volume, (4) edition, (5) name of publisher, (6) place of publication, (7) year of publication, and (8) page number. A complete book reference may be illustrated as follows:

(1) Gilman, H., "Organic Chemistry," Vol. 2, 2nd ed., John Wiley and Sons, New York, 1943, pp. 1236, 1252.

Arabic numerals must be used to designate all definite weights, measurements, percentages, and degrees of temperature unless occurring at the beginning of a sentence, in which case the numeral and measurement are spelled out. Periods of time should be written in words. Unusual characters and Greek letters should be written plainly and legibly or should be explained by notes on the margin of the page. Fractions should be written with a slant line. Standard abbreviations should be used whenever weights and measures are stated in the metric system, e. g., 10 Gm., 2.5 cc., etc. The forms to be used are: cc., Kg., Gm., mg., mm., cm., and L.

Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5" x 11" manuscript size. They should be designed for reduction to the width of one column (2.75") or full page width (5.5"). Thus, height rather than width should be accentuated where possible.

Numbers and measurements, etc., which are to appear on the axes of the drawing should be indicated in pencil outside the axes, in order that they may be set in type. Captions and legends should be placed *below* the diagram (not on it), or, if lengthy, collected on a separate typewritten list, identifying each by its proper figure number and including whatever acknowledgments are necessary.

Photographs should never be rolled, and paper clips should not be used unless guarded by a piece of paper folded several times to prevent an indentation in the photograph. All drawings and photographs should be protected from bending and from the chance of having their edges torn while in transit. Do not write on the back of a photograph. Any indentation in the photograph, however slight, will be likely to show as a blemish in the reproduction. All figures submitted must be referred to in the text of the manuscript, and should be numbered consecutively with Arabic numerals, e. g., Fig. 1, Fig. 2, etc. Kymograph tracings should not be submitted with the original experimental legends which are likely to have been hastily or carelessly inscribed. Authors may find it worth while to employ a professional draftsman to make the drawings in proper form for reproduction.

Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be constructed so as to occupy fully a single column (2.75") which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretative statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript. Every table and every column should be supplied with an appropriate heading. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

Your Son and Daughter in Your Pharmacy

insure for them interesting and successful careers in prescription, hospital and manufacturing pharmacy through degree courses at this institution, the oldest, yet most modern of its kind in the Americas. Studies lead to B.Sc., M.Sc. and D.Sc. degrees. Schools of Chemistry, Biology and Bacteriology. Since 1821. Write for free catalog.

***Philadelphia College* OF PHARMACY AND SCIENCE**

43rd St., Woodland & Kingsessing Aves., Philadelphia 4, Penna.

American Institute of the History of Pharmacy

George Urdang, Director, 457 Chemistry Building, Madison 6, Wisconsin.

( ) I wish to receive the Institute's publications and to support its work through membership. $5.00 dues are inclosed.

( ) Please send free pamphlet telling about benefits of membership and services of the Institute.

NAME_____

STREET ADDRESS_____

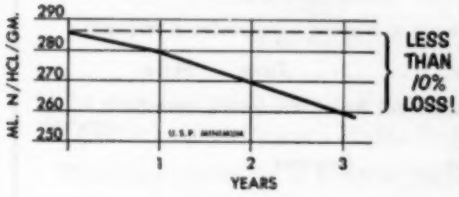
CITY_____ ZONE_____ STATE_____

REHEIS

$Al(OH)_3$ DRIED GELS LOSE LESS THAN 10%

ACID-CONSUMING CAPACITY IN THREE YEAR AGING TEST*!

Actual measured values:



F-2000 Powdered Gel Aging Test for Acid-Consuming Capacity

At start of test	286
After 1 year	279
After 2 years	270
After 3 years	260

*Conducted according to U.S.P. XIV acid-consuming capacity test procedure

This is just one *more* reason why REHEIS is the standard of quality in dried and compressed Al(OH)₃ gels.

Write for data and samples ...just clip this ad to your signed letterhead.

REHEIS COMPANY, INC.
Manufacturers of Fine Chemicals
BERKELEY HEIGHTS NEW JERSEY

Analyses · Consultation · Research

New Drugs

Bio-assays · Toxicological Studies
Formula Development · Special Analyses
Litigation · Phenol Coefficients

LaWall & Harrisson

Div. P, 1921 Walnut St., Philadelphia 3, Pa.

Bacteriologists · Chemists · Pharmacologists

Handsome Durable Binders for Journals of the American Pharmaceutical Association

Combination Sets for Practical Pharmacy and Scientific Editions $5.50 a pair
For Single Editions $3.25 each

These binders open flat as a bound book. Made of durable imitation leather, they will preserve your journals permanently. Each cover holds 12 issues (one volume)....Do your own binding. Instructions easy to follow. Mail coupon for binders on 10 day trial.

.....Mail Coupon Today.....

SUCKERT LOOSE-LEAF COVER CO.
234 W. LARNED ST. DETROIT 26, MICH.

Send....binders for the Journal of the American Pharmaceutical Association.
....Practical Pharmacy Edition for years....Vols.....
....Scientific Edition for years.....Vols.....
....Combination for years.....Vols.....
Will remit $..... in 10 days or return binders.
Name .....
Address .....
City.....Zone.....State.....
Postage prepaid in U. S.—Foreign Extra

DRUG STANDARDS

A Publication of
The American Pharmaceutical Association

Single copies $1.00
Annual Subscription $5.00

Order from

The American Pharmaceutical Association
2215 Constitution Avenue
Washington 7, D. C.

Rec'd_____ No._____
(DO NOT FILL IN) (DO NOT FILL IN)

Application for Active Membership in the American Pharmaceutical Association

Approving of its objectives, I hereby apply for ACTIVE MEMBERSHIP in the *American Pharmaceutical Association* and I enclose $15.00 to cover annual dues for the twelve-month period beginning with the month following my election to membership. Six dollars of the dues is for a year's subscription to the *Journal of the American Pharmaceutical Association*, which is published monthly in two editions, "*Practical Pharmacy Edition*" and "*Scientific Edition*." Annual subscription to the *Journal of the American Pharmaceutical Association* for nonmembers is $5.00 for the *Practical Pharmacy Edition*, $6.00 for the *Scientific Edition* or $10.00 combined.

Name in Full: Miss / Mr. / Mrs._____
(PLEASE PRINT OR TYPE NAME AND ENTIRE APPLICATION IF POSSIBLE)

Street and No._____ City_____ Zone____ State_____

Date of Birth_____ Of what State Pharmaceutical Assn. are you a member?____
(STATE)

Graduate of_____ Year_____ Degrees_____
(INSERT NAME OF COLLEGE)

Registered Pharmacist in_____
(INSERT NAMES OF ALL STATES IN WHICH REGISTERED)

Please indicate by check mark below your classification or classifications according to present employment:

____Retail Pharmacist (R)
____Hospital Pharmacist (H)
____Government Pharmacist (G)
____Teacher (T)
____Wholesaler (W)
____Manufacturer (M)
____Representative (Rp)
____Research or Control Chemist (Rcc)
____Association Official (A)
____Student (S)
____ _____
____ _____
(OTHER)

Signature of Applicant_____

This application is endorsed by the following members of the *American Pharmaceutical Association:*

Name_____ Name_____

Please send this application with check or money order for $15.00 to:

ROBERT P. FISCHELIS, Secretary,
American Pharmaceutical Association
2215 Constitution Avenue N. W., Washington 7, D. C.

M_____
CO_____
C_____
CIR_____

101st Convention

American Pharmaceutical Association

and

Affiliated and Related Organizations

Hotel Statler
Boston, Mass.
August 22-27, 1954

(Hotel Reservation Form on following page)

The next annual meeting of the American Pharmaceutical Association will be held in Boston, Mass., August 22 to 27, 1954.

Members planning to present papers before any Section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly.

Titles and abstracts *(*not to exceed 200 words*)* of papers should be submitted by *May 15, 1954, or preferably earlier,* in order to insure a place on any section program. Send titles and abstracts of papers to the appropriate Section Secretary.

SCIENTIFIC SECTION
Ray S. Kelley, Secretary
179 Longwood Avenue
Boston 15, Mass.

EDUCATION AND LEGISLATION SECTION
Frank L. Mercer, Secretary
St. Louis College of Pharmacy and Allied Sciences
Euclid and Parkview Place
St. Louis 10, Mo.

HISTORICAL PHARMACY SECTION
George E. Osborne, Secretary
2488 Redondo Avenue
Salt Lake City, Utah

PHARMACEUTICAL ECONOMICS SECTION
James W. Lansdowne, Secretary
5235 Cornelius Avenue
Indianapolis 8, Indiana

PRACTICAL PHARMACY SECTION
Samuel W. Goldstein, Secretary
2215 Constitution Avenue, N. W.,
Washington 7, D. C.

For detailed information address the

Secretary of the American Pharmaceutical Association, 2215 Constitution Avenue, N. W., Washington 7, D. C.

HOTEL RESERVATION FORM

1954 Convention

AMERICAN PHARMACEUTICAL ASSOCIATION

August 23–27, 1954 Boston, Massachusetts

NO RESERVATIONS WILL BE ACCEPTED DIRECTLY BY HOTELS. Use the following form in making all reservations.

A. PH. A. HOUSING COMMITTEE
Boston Convention and Visitors Bureau
80 Federal Street
Boston 10, Massachusetts

Please make hotel reservation(s) as shown below:

HOTEL.....First Choice

HOTEL.....Second Choice

HOTEL.....Third Choice

Number of room(s) with bath.....Double room(s).....Rate preferred $.....

.....Twin Bedded room(s).....Rate preferred $.....

.....Single room.....Rate preferred $.....

Arriving August.....a.m.....p.m .....Leaving August.....

A "double" room contains only one bed for occupancy by two persons.

THE NAME OF EACH HOTEL GUEST MUST BE LISTED

Please print the names of both persons for each double room or twin bedded rooms requested. Also names of all persons for whom you are requesting reservations and who will occupy the rooms asked for. OTHERWISE THE APPLICATION WILL BE RETURNED.

.....

.....

.....

APPLICANT..... STREET ADDRESS.....

CITY.....ZONE..... STATE.....

HOTEL RATES IN BOSTON

HOTEL	Singles	Doubles	Twins	Suites
Statler Hotel (Headquarters)	6.00–11.00	10.00–14.00	11.50–17.00	26.00–30.50
Bellevue Hotel	5.00– 7.50	7.50–10.00	9.50–16.00	
Bradford Hotel	4.95– 6.75	7.75– 9.50	9.50–13.00	14.00–18.00
Kenmore Hotel	5.50– 9.00	9.00–12.50	9.50–13.00	14.00–20.00
Lenox Hotel	4.50– 6.00	6.50– 8.50	10.00	11.00–16.00
Manger Hotel	5.00– 7.50	7.50– 9.50	9.00–11.00	
The Parker House	6.00– 8.75	9.50–10.00	11.00–14.00	23.00–25.00
Sheraton Plaza Hotel	5.85 up	9.00 up	11.00 up	25.00 up
Somerset Hotel	7.00–10.00	10.00–14.00	12.00–15.00	18.00–40.00
Touraine Hotel	5.50– 8.00	8.00–10.00	8.50–12.00	18.00
Vendome Hotel	4.50– 6.00	7.00–10.00	8.00–11.00	12.00–20.00

MAIL REQUEST FOR ROOM RESERVATIONS TO:

A. PH. A. HOUSING BUREAU

Boston Convention and Visitors Bureau
80 Federal Street, Boston 10, Massachusetts

Superior Ingredients...

The AMERCHOLS® are natural, non-ionic OINTMENT BASES AND EMULSIFIERS which contain *Lanolin Cholesterols in their Most Active Form.*

These stable cholesterol and multi-sterol surfactants induce rapid drug release, promote optimum healing rates, and are safe for the most delicate tissues. We know of no case of an allergy due to an AMERCHOL.*

**References, technical data and suggested formulations are available from our research laboratories. Please write on your business letterhead.*

For superior products

MODULAN® is a chemically modified LANOLIN EMOLLIENT with unique new properties for use in OINTMENTS AND EMULSIONS. It imparts waxy, protective hydrophobic films; is oil soluble and compatible with O/W emulsions, soaps and shampoos. Clinical investigations indicate that MODULAN IS HYPO-ALLERGENIC.*

AMERICAN CHOLESTEROL PRODUCTS
· INCORPORATED ·
MILLTOWN — NEW JERSEY

Scale illustrated is our No. 540 all Glass Model

Capacity—4 oz. (120 grams)
Sensitivity—1/10 grain
Finish—Genuine light mahogany with chrome plated parts
Price—$145.00 (subject to change without notice)

PROMPT DELIVERY on this as well as other class A scales

No. W35 (all glass in lid) $110.00
No. W33 ..... 98.00
All With Stainless Steel Pans

Our scales have no bands or wires to stretch or break

Still the best and lowest priced scale made

HENRY TROEMNER

Manufacturer of the Finest Scales for the Druggist Since 1840

911 Arch Street
Philadelphia 7, Pa.
(Send for Circular A-A)

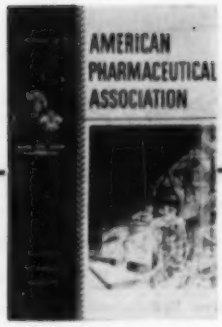
The Massachusetts College of Pharmacy

Longwood Avenue, Boston

Graduate study in Boston has always appealed strongly to students in the fields of medicine and public health. The environment is stimulating and attracts students from all parts of the world. Library, laboratory, hospital, and other facilities for graduate work are outstanding in this area.

The Massachusetts College of Pharmacy is located in this great medical and educational center. It offers unusual opportunities to graduates of colleges of pharmacy who are interested in further study in pharmacy, pharmaceutical chemistry, and pharmacognosy.

For additional information, write to HOWARD C. NEWTON, Dean



A Preview of Advertising

In the March 1954 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ **Erythrocin Stearate** and **Iberol** tablets are **Abbott Laboratories'** current features.

▸ **Span, Tween, Arlacel** and **Sorbo** are being put to "ever-widening" uses in pharmaceutical preparations, the **Atlas Powder Company** reports.

▸ Its soluble sulfonamide, **Gantrisin,** is the subject of the **Hoffmann-LaRoche Inc.** insertion.

▸ The established value of **Thantis Lozenges** as an anesthetic-antiseptic for throat irritations is emphasized by **Hynson, Westcott & Dunning.**

▸ **Lederle Laboratories Division,** American Cyanamid Co., reports that **Achromycin,** its new broad-spectrum antibiotic, has the advantages of more rapid absorption, quicker diffusion in tissue and body fluids, and increased stability.

▸ "A more intense antibacterial effect" is **Eli Lilly & Company's** description of the action of **'Ilotycin-Sulfa'.**

▸ The **S. E. Massengill Company** lists the following prescription specialties in its announcement: **Adrenosem, Aminodrox, Broniacin, Livitamin, Massengill Powder, Obedrin, Salcedrox,** and **Semhyten.**

▸ ". . . and be sure to take your Vitamins!" admonishes **Merck & Company.**

▸ **Duraglas** prescription containers are featured by **Owens-Illinois.**

▸ **Milontin,** for use in the treatment of epilepsy, is advocated by **Parke, Davis & Co.**

▸ From **Pfizer Laboratories** come timely announcements of **Combandrin, Cortril** tablets (adrenocortical hormone for systemic use) and **Terramycin** for oral, as well as intramuscular use.

▸ Studies at the **Philadelphia College of Pharmacy and Science** lead to B.Sc., M.Sc. and D.Sc. degrees. The College also has schools of chemistry, bacteriology, and biology.

▸ The **A. H. Robins Company** focuses attention on its product, **Donnatal,** a sedative-antispasmodic, cholinergic depressant.

▸ Its antibiotic, **Tetracyn, J. B. Roerig & Company** declares, is a profit builder for the prescription department.

▸ **Chlor-Trimeton** injection "100"—for the control of symptoms in allergic manifestations—is offered by **Schering Corporation.**

▸ **Sharpe & Dohme** suggests **Tracinets** for throat irritations and **Penalev** to make oral penicillin therapy acceptable to children as well as adults.

▸ Dispensers of **Benzedrex Inhalers (Smith, Kline & French Laboratories)** report quantity sales.

▸ **E. R. Squibb & Sons** calls attention to **Novogran,** for use in periods of physiological stress.

▸ **Methischol** is described by **U. S. Vitamin Corporation** as "the complete lipotropic therapy" in atherosclerosis, coronary disease, diabetes, and obesity.

▸ **Peritrate** for angina pectoris is offered by **Warner-Chilcott Laboratories.**

▸ For common colds, allergic rhinitis, and sinusitis, **Winthrop-Stearns Inc.** advocates its new **NTZ Nasal Spray.**

▸ **Wybiotic** is **Wyeth Laboratories'** new triple antibiotic for infections.

Look for Details in the Practical Pharmacy Edition of This Journal—Out March 20

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIII — *MARCH, 1954* — NUMBER 3 — CONSECUTIVE NO. 6

The Kinetics of Degradation of Chloramphenicol in Solution*

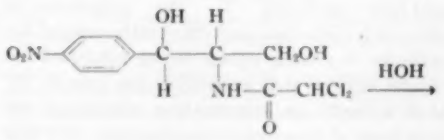
II. Over-all Disappearance Rate from Buffered Solutions

By TAKERU HIGUCHI, ARNOLD D. MARCUS, and CHARLES D. BIAS†

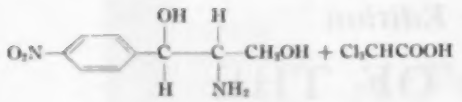
The rate of disappearance of chloramphenicol molecules, *per se*, from aqueous solution has been studied and shown to be first order over a wide range of hydrogen-ion concentration. The data obtained indicate rather clearly that the rate of degradation is independent of the ionic strength of the medium employed for degradation. In addition, the rate of degradation appears to be substantially independent of the hydrogen-ion concentration of the system within the *p*H range 2–7. Very strong evidence exists to indicate that chloramphenicol degradation is general acid-base catalyzed. For example, monohydrogen phosphate ions, undissociated acetic acid molecules, and citrate–citric acid systems exert a definite catalytic effect upon the rate of antibiotic disappearance. The last example shows a rather complex relationship which might possibly be ascribed to the polybasic nature of citric acid and its ions, in which both electrophilic and nucleophilic functions are present within the same molecule.

IN THE PRECEDING communication of this series (1), the mechanism and the influence of several variables upon the rates of the dehalogenation reactions responsible, in part, for the gradual loss of activity of chloramphenicol in aqueous solutions were treated and the degradative rate was observed experimentally by following the rate of chloride ion production in the aqueous solutions. In contrast, in the present investigation we were concerned primarily with the total rate of disappearance of chloramphenicol molecules, *per se*, from the reaction system, the analytical scheme employed being designed to determine the residual concentration of the intact drug rather than any of its degradative products (2). From the experimental data presented, the dehalogenation reaction undergone by chloramphenicol appears to play but an insignificant part in the total degradative picture, at least in solutions below *p*H 7.

In the study referred to above (1), kinetic analysis indicated that one of the major causes of chloramphenicol breakdown can be attributed to the hydrolytic cleavage of the amide linkage in the drug according to the equation



* Received August 21, 1953, from the School of Pharmacy, University of Wisconsin.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
This project was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and in part by a grant from Parke, Davis & Co., Detroit, Mich.
† Parke, Davis Fellow, University of Wisconsin.



Subsequent observations on the over-all disappearance rate appear to substantiate further this hypothesis, as the rate is highly reproducible under any given experimental conditions and is strictly first order with respect to the drug. These are characteristics of molecular reactions of the hydrolytic type as contrasted to redox reactions which are free radical mediated and rather difficult to reproduce.

Even if the degradative mechanism is assumed to be hydrolytic cleavage of the amide function, only a relatively small amount of help can be gained by reference to the literature for comparable reactions. Although a considerable amount of research effort has been devoted to the fundamentals of the kinetics of hydrolytic cleavage of esters, very little work seems to have been done on the similar cleavage of amides. This is probably due to the fact that amides, as a whole, are a great deal more stable than esters.

Certain theoretical assistance can be gained, however, by reference to the general theory of acid-base catalyzed reactions. In many instances the hydrolytic cleavage of ordinary esters and amides is catalyzed by hydrogen ions, hydroxyl ions, or both. The hydrolysis of esters such as ethyl acetate, aspirin (3), and procaine (4) appears to be catalyzed by hydrogen ions or hydroxyl ions alone (i. e., specific hydrogen or hydroxyl ion catalysis). The hydrolysis of such simple amides as formamide and acetamide has been studied and shown to be catalyzed by hydrogen and hydroxyl ions (5, 6). In view of these instances of catalysis, it appeared rather likely that the amide linkage in chloramphenicol may also be subject to hydrogen or hydroxyl ion-catalyzed hydrolysis.

If we are to assume that hydrogen and hydroxyl ions are not unique as acidic or basic species but that other species may also be considered to function as acids and bases, a new factor is introduced in considering the kinetics of hydrolytic reactions including degradation. It has been recognized in a number of instances that hydrolysis may be catalyzed by acids and bases other than hydrogen and hydroxyl ions (i. e., general acid-base catalysis) (7). The hydrolysis of ethyl orthocarbonate and ethyl orthoacetate, for example, has been shown to be catalyzed by general acids as well as by hydrogen ions (8, 9). More recently an investigation concerning the hydrolysis of diisopropyl fluophosphate revealed that hydrolysis of this compound is also general acid-base catalyzed (10). Among well-known instances of nonhydrolytic general acid-base catalyzed reactions are the mutarotation of glucose and the decomposition of diazoacetate ion (11, 12).

Because of the several possible pathways by which amide cleavage may be effected, attempts have been made in the present investigation to study the kinetics of chloramphenicol breakdown as influenced by neutral ions, hydrogen ions, hydroxyl ions, general acids, and general bases. Indeed, it was difficult to limit attention to hydrogen or hydroxyl ions alone, inasmuch as the maintenance of a constant *p*H in the degradative systems necessitated the use of buffers which by their very nature contain significant amounts of general acids and general bases.

The results of the present study indicate that degradation of the antibiotic is general acid-base catalyzed but that within the *p*H range 2–7 the rate of degradation is substantially independent of hydrogen-ion concentration.

EXPERIMENTAL

Reagents.—All reagents used in this investigation were of analytical grade. Silicic acid, chromatography grade, was used to prepare the columns. The chloramphenicol was eluted from the columns with chloroform and a 10% (v/v) solution of ethyl acetate in chloroform. The buffers referred to below were prepared by dissolving reagent grade chemicals in distilled water, adjusting, and measuring the *p*H with a Beckman Model G *p*H Meter.

Procedure.—Chloramphenicol, 250 mg., was accurately weighed into 100-ml. volumetric flasks with the aid of a small glass funnel. Any of the compound adhering to the sides of the funnel was washed into the flask with approximately 90 ml. of the appropriate buffer solution. The flasks were then placed into a constant temperature bath at 97.30 ± 0.05° and the contents allowed to attain the temperature of the bath. At that point the contents of the flasks were made up to the mark with the proper buffer solution and the flasks were securely stoppered. The first sample was withdrawn from each of the reaction vessels only after visible inspection showed that all of the chloramphenicol in each flask had dissolved and the timing of the reaction was then started. Samples, approximately 5 ml. in volume, were withdrawn with volumetric pipets at appropriate intervals, placed into glass vials (8-ml. capacity), and protected from the cap of the vial with aluminum foil.

The concentration of chloramphenicol in each sample was determined by partition chromatographic separation and subsequent spectrophotometric analysis according to a procedure reported elsewhere (2).

DISCUSSION OF EXPERIMENTAL RESULTS

Sufficient experimental data have been collected in the present investigation to determine the order

of the reaction with respect to chloramphenicol and to ascertain the relative catalytic activities of hydrogen ions, hydroxyl ions, and certain general acids and bases within a *p*H range of 2–7.

Order of the Deterioration Reaction with Respect to Chloramphenicol.—As mentioned in the introduction, the rate of antibiotic disappearance from buffered aqueous solutions appears to be strictly first order with respect to antibiotic concentration. This relationship is apparently valid over widely differing hydrogen-ion concentrations as is evident from Fig. 1. The linear logarithmic plots have been found to be extremely reproducible under all encountered experimental conditions and in no case have they deviated significantly from the over-all first-order relationship.

In much of the following discussion the rate of antibiotic disappearance is expressed in terms of the half-life of the drug because of the direct dependency of the deterioration rate upon the concentration of chloramphenicol. In certain instances, however, it was found convenient to make use of reciprocal half-lives rather than the half-lives themselves. This usage is particularly useful in determining the effect of general acid-base catalysis upon the rate of chloramphenicol disappearance. It may be seen from the relationship $k = 0.693/\text{half-life}$, where k is the first-order rate constant, that the value of the rate constant, in reciprocal time, is directly proportional to the reciprocal of the half-life.

The absolute rate of chloramphenicol disappearance as determined from the plots shown in Figs. 3, 4, and 5 indicates that the dehalogenation reactions contribute to a very minor extent in the *p*H interval 6–7 and negligibly below *p*H 6.00, to the over all reaction. This is based on the fact that the maximum half-life of chloramphenicol at 97.30° was fourteen hours, whereas the minimum half-life based on the dehalogenation reaction was over one hundred hours at *p*H 7 and much longer at lower hydroxyl-ion concentrations (1).

Ionic Strength Independence of Degradation Rate.—The lack of a formal charge on the chloramphenicol molecule would make it seem unlikely that the ionic strength of the buffers employed for degradation would be of significance in affecting the rate of degradation. However, in view of the partial ionic character of the nitro group and the fact that one of the resonance forms of an amide will show a separation of charge, it was thought that any dependency of the degradation rate upon ionic strength should be determined, so as to facilitate proper evaluation of data obtained in presence of buffers.

Degradation of chloramphenicol was therefore carried out at 97.30° in 0.062 *M* phosphate buffer adjusted to *p*H 7.00 and the half-life thus obtained was compared with that obtained with the same buffer containing, in addition, 60 Gm. of sodium sulfate per liter. The difference in the ionic strengths of the two systems was such that any dependency of the degradative rate upon the ionic strength of the medium would be indicated after analysis of the samples. As shown in Fig. 2, practically identical results were obtained from both systems, thus indicating that the rate of degradation is essentially independent of ionic strength. In both instances the half-life of the antibiotic was 7.95 hours.

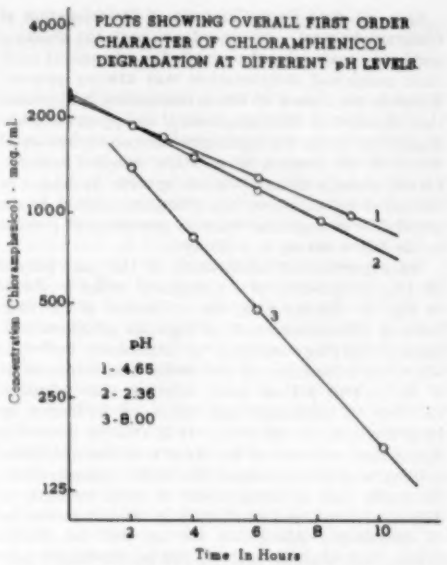


Fig. 1.—Plots showing the over-all first order character of chloramphenicol degradation in different buffers. The *p*H values given above, 2.36, 4.65, and 8.00, were maintained by the use of 0.50 *M* citrate, 1.50 *M* acetate, and 0.062 *M* phosphate buffers, respectively.

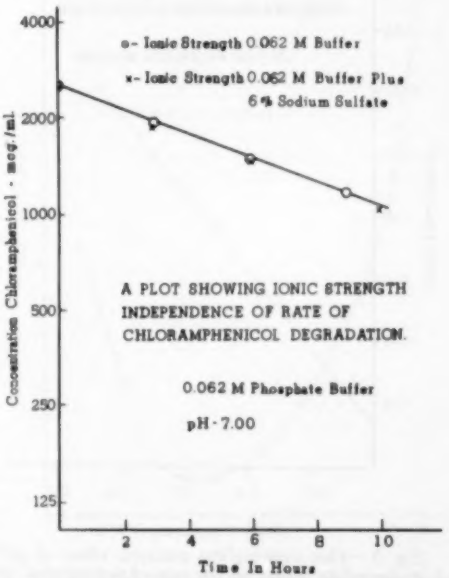


Fig. 2.—Plot showing the rate of chloramphenicol degradation in 0.062 *M* phosphate buffer and in the identical buffer containing, in addition, 6% sodium sulfate. The coincidence of the experimentally determined points shows that the rate of chloramphenicol degradation is essentially independent of ionic strength.

General Acid-Base Catalysis of Deterioration of Chloramphenicol.—In accordance with the thinking outlined previously, the possibility of general acid-base catalyzed deterioration was always present. Early in the course of the investigation it appeared that the rate of chloramphenicol disappearance was dependent upon the hydrogen-ion concentration of the buffered systems in a rather complex manner. In the absence of any notable specific hydrogen or hydroxyl ion catalysis, the evidence pointed to the possibility of a general base or general acid present in the buffer acting as a catalyst.

An experimental verification of the part played by the components of a phosphate buffer is shown in Fig. 3. In the plot, the reciprocal of the half-lives of chloramphenicol in aqueous solutions containing varying amounts of phosphate buffer is shown as a function of the buffer concentration at 97.30°. The *p*H of each solution was adjusted to 7.00 to minimize any effect of hydrogen or hydroxyl ions on the rate. It is evident from this figure that the rate of breakdown of the antibiotic is linearly dependent upon the buffer concentration. Since the rate is independent of ionic strength as discussed above and unaffected by the concentration of dihydrogen phosphate ion as will be shown below, this catalytic activity can be attributed only to monohydrogen phosphate ion acting as a general base. This is quite surprising in view of the relatively weak nucleophilic character of this ion.

To test the possibility that dihydrogen phosphate ion was acting as a catalyst of the general acid

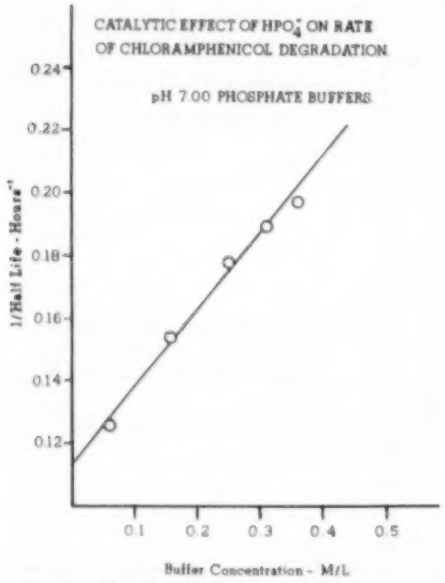


Fig. 3.—Plot showing the catalytic effect of *p*H 7.00 phosphate buffer on the rate of degradation of chloramphenicol. In view of the data shown in Table I, the catalytic activity of this buffer mixture may be properly ascribed to the monohydrogen phosphate ion. Extrapolation of the experimentally determined values to zero buffer concentration indicates the rate of chloramphenicol degradation at *p*H 7.00 and zero buffer concentration.

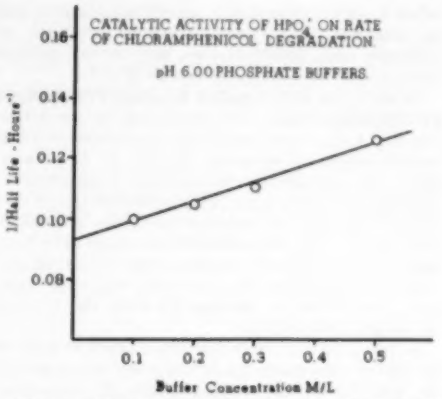


Fig. 4.—Plot showing the catalytic effect of *p*H 6.00 phosphate buffers on the rate of degradation of chloramphenicol. Extrapolation of the experimentally determined points to zero buffer concentration is indicative of the rate of degradation at *p*H 6.00 and zero buffer concentration.

type, the deterioration rate was studied in a series of solutions containing the same concentration of HPO_4^{--} (0.2 *M*) but varying amounts of $H_2PO_4^-$. Although the concentration of the acidic component was changed over a large range as reflected in the *p*H of the solution, the resulting half-life values remained essentially constant as shown in Table I. It is evident from these data that $H_2PO_4^-$ has little or no catalytic activity under the conditions employed for this study, the catalytic behavior of the entire buffer being attributable only to the doubly charged ion.

TABLE I.—RATE OF CHLORAMPHENICOL DEGRADATION IN PHOSPHATE BUFFERS 0.2 *M* WITH RESPECT TO MONOHYDROGEN PHOSPHATE ION AND CONTAINING VARYING AMOUNTS OF DIHYDROGEN PHOSPHATE

*p*H	Half-Life Hours
6.98	5.3[a]
6.77	6.2
6.61	6.2
6.24	6.1
5.95	6.3

[a] The discrepancy introduced by this value, substantially below the others, may be ascribed almost entirely to hydroxyl-ion catalysis of the dehalogenation reaction referred to in the introduction.

A similar investigation indicated that acetate buffers also act as catalysts for the deterioration of the antibiotic. In Fig. 5, where experimentally determined reciprocal half-lives have been plotted against total acetate concentration, i. e., the sum of the concentrations of equimolar sodium acetate and acetic acid, it is evident that the buffer exerts a small but definite catalytic effect on the over-all rate. Unlike the phosphate systems, however, further studies have shown that the catalytic activity is associated almost entirely with the acidic component of the buffer and that the conjugate base possesses only slight catalytic activity. Thus, as is shown in Table II, in systems containing constant

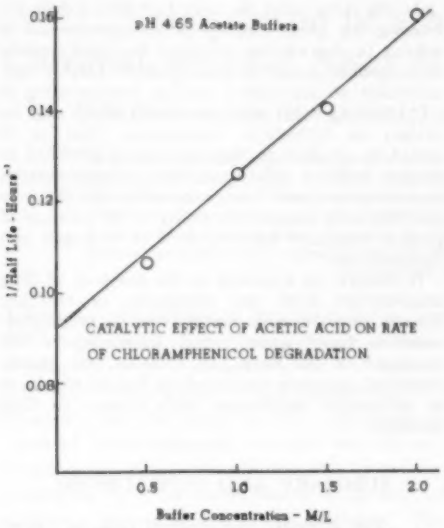


Fig. 5.—Plot showing the catalytic effect of undissociated acetic acid upon the rate of chloramphenicol degradation. The reciprocal half-life obtained at the point of intersection of the straight line with the ordinate is identical with the value for the reciprocal half-life at zero buffer concentration and *p*H 6.00, thus showing a substantial degree of *p*H independence of the degradation reaction.

amounts of acetic acid, variations in the acetate ion concentration have relatively little effect on the rate of degradation. These data suggest that the catalytic behavior is due essentially to *general acid* catalysis with but a small contribution resulting from general base catalysis.

TABLE II.—RATE OF CHLORAMPHENICOL DEGRADATION IN ACETATE BUFFERS 0.05 *M* WITH RESPECT TO ACETIC ACID AND CONTAINING VARYING AMOUNTS OF SODIUM ACETATE

*p*H	Sodium Acetate Concentration	Half-Life, Hours
5.95	1.0 *M*	10.9
		11.0
		11.1
4.87	0.10 *M*	13.4
		13.3
4.58	0.05 *M*	10.9
		14.0
		13.9

Although the catalytic properties of both phosphate and acetate systems can be ascribed to their acid-base characteristics, it is important to keep in mind that the effect may be a specific property of the catalytic species and only indirectly related to their acid-base character. This possibility is partially supported by a study of the degradative rate in presence of citrate buffers. Unlike the acetate or phosphate system, citrate systems present the possibility of having more than one acidic or basic species in the buffer solution; thus there may be present

Citric acid
Dihydrogen citrate ion
Monohydrogen citrate ion
Citrate ion

all in equilibrium and possessing differing catalytic tendencies. This rather complex system can be expected to, and does, result in an involved dependency of the catalytic effect upon the hydrogen-ion concentration although the degradative reaction itself is not specifically influenced, within this *p*H range, by hydrogen ions (see below). The apparent dependency of the reaction rate, as expressed in half-lives, on the hydrogen-ion concentration is shown in Fig. 6. It is evident that at higher *p*H values the half-life is essentially unaffected by the buffer since the values are close to the value of eleven hours. In the *p*H region immediately below this, we find a rapid increase in the reaction rate which can be ascribed to the catalytic effect of the singly charged dihydrogen citrate and/or the doubly charged monohydrogen citrate. The minimum exhibited by the curve at approximately *p*H 3 and the very sharp reversal of the reaction rate appear difficult to rationalize. It is possible that either the singly or doubly charged ion or both possess significant catalytic activity but that the triply charged citrate ion and undissociated acid do not. These differences are difficult, however, to explain on the basis of general acid-base catalysis alone.

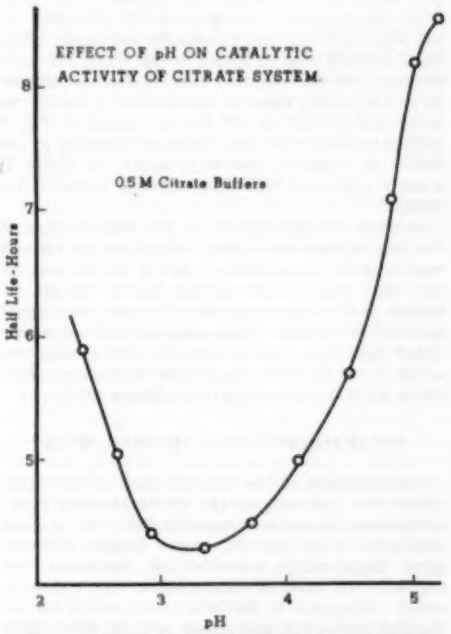


Fig. 6.—Plot showing the effect of hydrogen-ion concentration upon the ability of a citrate buffer system to catalyze the degradation of chloramphenicol. The shape of the curve together with the position of the minimum indicate that the effective catalytic species in the citrate buffer systems are monohydrogen citrate ion and/or dihydrogen citrate ion.

A possible explanation of the unusual behavior of the citrate systems may be found in the suggestion by Swain (12) that all reactions of this type (i. e., general acid-base catalyzed) are termolecular in nature and involve both electrophilic and nucleophilic catalysts; the exceptional catalytic efficacy of the partially neutralized citrates being due, on this basis, to the dual nature of these acidic salts.

pH Independence of Rate of Chloramphenicol Degradation over pH Range 2–7.—In the preceding two sections we tacitly assumed that the rate of the deterioration reaction was independent of hydrogen- or hydroxyl-ion concentration, i. e., not subject to specific hydrogen or hydroxyl ion catalysis. This assumption was based on the fact that the reaction rate in absence of any interfering buffer was found to exhibit *p*H independence.

Although it is impossible from a practical standpoint to determine directly the rate of the reaction at any given *p*H in absence of buffer because of the acidic nature of the reaction products, the corresponding rate can be obtained by extrapolation to zero buffer concentration of a series of results determined in presence of buffers of varying concentration. Thus in Fig. 3 the intersection of the straight with the ordinate corresponds to the reciprocal half-life at *p*H 7.00 and zero buffer concentration. In Fig. 4 a similar plot is shown for a series of experimental determinations at *p*H 6.00. If the rate of chloramphenicol disappearance due to the hydroxyl-ion catalyzed dehalogenation reaction at *p*H 7.00 is subtracted from the over-all rate of antibiotic disappearance at the same *p*H, the values at *p*H 6.00 and 7.00 are essentially the same, being equal in each case to eleven hours. The *p*H independence of the reaction rate is further demonstrated by noting that the extrapolated value for the reciprocal half-life at *p*H 4.65 as shown in Fig. 5 is identical with the value obtained similarly at *p*H 6.00. In addition, the data shown in Table I, when regarded in this light, strongly support this view.

Further confirmation of the *p*H independence of the degradation rate within the *p*H range studied may be gained by reference to Fig. 6. It may be seen that extrapolation of this plot to the *p*H extremes of the citrate systems employed once again give half-life values of approximately eleven hours. Other data have shown that the deterioration reaction is not significantly affected by hydrogen ions above *p*H 2 nor by hydroxyl ions below *p*H 7.

PHARMACEUTICAL SIGNIFICANCE

Consideration of the experimental evidence presented can lead only to the conclusion that chloramphenicol possesses unusual stability in aqueous solutions, over a wide *p*H range. Despite the very great temperatures to which the antibiotic was exposed, the rate of degradation was relatively small. Inasmuch as the heat of activation for the dehalogenation has been shown to be 30,000 calories per mole (1) and the indications, based on additional experimental determinations, that the heat of activation for the postulated amide hydrolysis is of the order of 23,000 calories per mole, it is readily evident that within the *p*H range 2–7 the antibiotic shows almost no susceptibility to hydrolytic deterioration at ordinary temperatures.

At the same time, the very fact that a drug exhibiting the great stability of chloramphenicol is subject to degradation catalyzed by buffer constituents should point the way toward careful consideration of any buffers used in compounding or administering other pharmaceuticals which may be subject to hydrolytic degradation. Just as it would be prudent to dispense chloramphenicol in weakly buffered solutions, other pharmaceuticals possessing the amide linkage should be regarded very carefully with respect to stability in the presence of general acids and bases as well as hydrogen and hydroxyl ions.

In essence, an approach to the problems of drug deterioration from the standpoint of chemical kinetics provides very useful clues to proper formulation based upon actual knowledge of the character of the drug. In contrast, the purely empirical approach may overlook factors which are of substantial significance with respect to drug stability.

SUMMARY AND CONCLUSIONS

1. The over-all degradation rate of chloramphenicol in aqueous solution has been shown to be first order with respect to the antibiotic over a wide range of hydrogen-ion concentration.

2. The reproducibility of the experimental results along with the strict adherence to first-order dependency indicate that in addition to hydrolytic cleavage at the carbon chloride bonds, similar cleavage occurs elsewhere in the molecule. This is assumed to be at the amide linkage.

3. The rate of chloramphenicol degradation is independent of the ionic strength of the medium employed for degradation and largely independent of hydrogen-ion concentration within the *p*H range 2–7.

4. Definite evidence has been accumulated to show that chloramphenicol deterioration is catalyzed by species which are general acids and bases. Specifically, monohydrogen phosphate ion, undissociated acetic acid, and both mono- and dihydrogen citrate ions catalyze the rate of chloramphenicol degradation.

REFERENCES

(1) Higuchi, T., and Bias, C. D., THIS JOURNAL, **42**, 707(1953).
(2) Higuchi, T., Bias, C. D., and Marcus, A. D., *ibid.*, **43**, 135(1954).
(3) Edwards, L. J., *Trans. Faraday Soc.*, **46**, 723(1950).
(4) Higuchi, T., Havinga, A., and Busse, L. W., THIS JOURNAL, **39**, 405(1950).
(5) Krieble, V. K., and Holst, K. A., *J. Am. Chem. Soc.*, **60**, 2076(1938).
(6) Taylor, T. W. J., *J. Chem. Soc.*, **1930**, 2741.
(7) Bell, R. P., "Acid-Base Catalysis," Oxford University Press, London, 1941, pp. 48–80.
(8) Bronsted, J. N., and Wynne-Jones, W. F. K., *Trans. Faraday Soc.*, **25**, 59(1929).
(9) Kilpatrick, M., and Kilpatrick, M. L., *J. Phys. & Colloid Chem.*, **53**, 1385(1949).
(10) Lowry, T. M., and Smith, G. F., *J. Chem. Soc.*, **1927**, 2539.
(11) King, C. V., and Bolinger, E. D., *J. Am. Chem. Soc.*, **58**, 1533(1936).
(12) Swain, C. G., *ibid.*, **72**, 4578(1950).

Comparison of a Microbiological and a Chromatographic Assay Method for Chloramphenicol*

By TAKERU HIGUCHI, ARNOLD D. MARCUS, and CHARLES D. BIAS†

It is shown in the determination of chloramphenicol that a microbiological method and a chromatographic method yield identical results when tested against partially degraded samples. The former is based on repression of growth as followed by Warburg manometry, and the latter on isolation of the antibiotic on a partition column and subsequent determination by ultraviolet spectrophotometry. Because of its greater precision and rapidity, the chromatographic method appears to be preferable for routine determinations.

DURING THE COURSE of an investigation into the kinetics of the disappearance of chloramphenicol from aqueous solutions, it became expedient to follow the residual concentration of intact chloramphenicol molecules. Although an excellent microbiological method was already available (1), it was thought desirable to develop a chemical assay for the antibiotic in the hope of achieving greater accuracy and precision. The present communication is concerned with such a chemical analytical method for the determination of chloramphenicol in the presence of its degradation products as compared with a new microbiological method based upon respiratory inhibition of a bacterial culture.

THE MICROBIOLOGICAL METHOD

Biological methods are, in a sense, ideally suited for evaluation of drug activity or concentration since the end-use activity is determined directly. Such procedures are usually insensitive to the presence of similar but inactive contaminants or degradation products. They are, in most cases, very sensitive and are capable of detecting drugs present in extremely minute concentrations. In spite of the specificity and sensitivity characteristic of these procedures, however, their adoption as analytical procedures is usually with reluctance and as a last resort because of their inherent low degree of precision.

In the present study the microbiological approach was mainly employed to validate the results obtained by the chemical method. Although the turbidimetric method proposed by Joslyn and Galbraith (1) would have been equally suitable for this purpose, a manometric procedure was developed separately because of its somewhat greater rapidity. This procedure is based in principle upon the inhibition of oxygen uptake by *Escherichia coli* in the presence of several solutions of chloramphenicol of known concentration, the inhibition produced by a sample of unknown concentration being compared against those shown by a series of standards.

* Received August 21, 1953, from the School of Pharmacy, University of Wisconsin, Madison.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
This project was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and in part by a grant from Parke, Davis & Co. of Detroit, Mich.
† Parke, Davis Fellow, University of Wisconsin.

THE EXPERIMENTAL METHOD

Apparatus.—Warburg constant volume respirometer.

Preparation of the Media.—(*a*) Difco nutrient agar (23 Gm.) is suspended in 100 ml. of freshly distilled water and the suspension is heated to boiling in order to effect solution; 25 ml. of the agar solution is then poured into ordinary prescription bottles which provide a fairly large surface for bacterial growth. (*b*) A synthetic glucose medium is prepared according to the method described by Kohn and Harris (2). (*c*) Both solutions are autoclaved at a pressure of 15 lb./sq. in. for twenty minutes and stored for use when necessary.

Preparation of the Culture.—A culture of *Escherichia coli* is transferred to a bottle of nutrient agar and incubated at 37° for twenty hours. The bacteria are then removed from the agar by washing with approximately 10 ml. of sterile distilled water and the resulting suspension is adjusted to an optical density of 1.0 (red filter) on a nephelometer. Five milliliters of this suspension are poured into a bottle containing 100 ml. of the synthetic glucose medium, incubated for one and one-half hours, and cooled in a refrigerator for one hour before use.

Preparation of Sample and Standard Solutions.—A standard solution of chloramphenicol containing 10 mcg./ml. is prepared by dissolving the drug in distilled water. Other samples of lower chloramphenicol content are prepared by proper dilution of this solution. The samples to be analyzed are weighed, dissolved, and diluted with distilled water so that the concentration of the antibiotic will fall within the range 5–10 mcg./ml.

Preparation of the Warburg Flasks.—Exactly 0.2 ml. of a 20% solution of potassium hydroxide is pipetted into the well of each Warburg flask and a 4-sq. cm. piece of fluted filter paper is inserted to absorb the alkali and to provide a large surface area for reaction with carbon dioxide. Two milliliters of the cold glucose medium containing the suspended bacteria are pipetted into each flask, the suspension being shaken before each withdrawal to obtain uniform inoculation in each of the several flasks. One milliliter of a standard chloramphenicol solution containing 5, 6, 7, 8, 9, or 10 mcg. of the antibiotic is added to each flask. In the same fashion, a 1-ml. aliquot of each sample solution is added to a separate flask. When higher accuracy is desired, duplicate or triplicate samples of the unknown solution are used.

The flasks are then attached to their corresponding manometers and placed in the water bath regu-

lated at 37°. The manometers are allowed to equilibrate thermally for ten minutes and are then closed to the atmosphere. Readings are taken every half-hour. From these readings the corresponding volume of oxygen uptake for the contents of each flask is calculated according to the procedure described by Umbreit (3).

Each "run" requires approximately eight hours including about four hours in the Warburg bath.

Calculations.—The oxygen uptake in microliters is plotted against the time (in hours) as shown in Fig. 1. A time is arbitrarily chosen during the logarithmic phase as a reference point. The oxygen uptake between this arbitrarily chosen time and zero time is then plotted against the logarithm of the chloramphenicol concentration of the standards as shown in Fig. 2. By interpolation from this graph the chloramphenicol contents of the unknown samples are obtained.

THE CHEMICAL METHOD BASED ON PARTITION CHROMATOGRAPHY

The chemical and instrumental methods of quantitative assay presently being employed are rather unspecific. Nearly all such procedures are essentially methods for determining the amounts of certain functional groupings present in the samples, very little differentiation being made, for example, between two alkaloidal nitrogens or between two carboxyl groups attached to different compounds found in the same sample. Thus in the analysis of chloramphenicol a number of analytical methods have been proposed, based on the presence of the *p*-nitrophenyl moiety in the molecule (4–6). It makes very little difference whether the grouping is determined by conversion to an azo compound or by polarography; they are still functional group analyses and not methods for determining chloramphenicol, *per se*. Such methods are, for example, unreliable in presence of the degradative products of the drug.

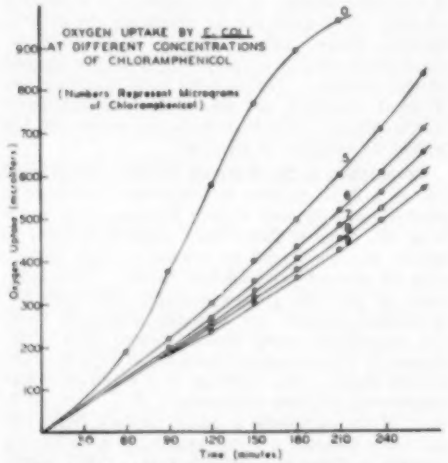


Fig. 1.—A plot showing the relationship between oxygen uptake by *E. coli* and time in the presence of varying amounts of chloramphenicol as determined by a Warburg Constant Volume Respirometer at 37° C.

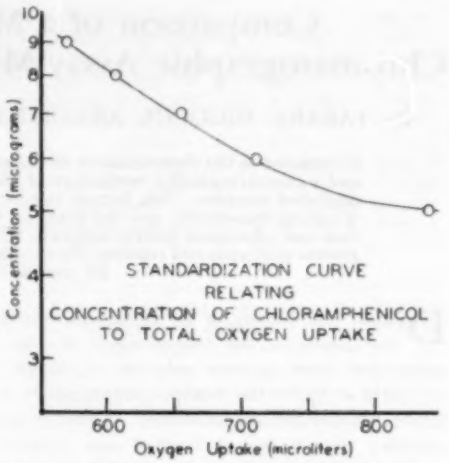


Fig. 2.—Plot showing the relationship between the logarithm of chloramphenicol concentration and the amount of oxygen consumed by *E. coli* as measured in a Warburg Constant Volume Respirometer at 37° C. This standard curve was employed in the microbiological procedure for determining the chloramphenicol content of unknown samples.

Because of the recent successes in the application of chromatographic methods to the analysis of complex pharmaceutical mixtures, an attempt has been made in this investigation to develop an analytical method for chloramphenicol based on isolation of the intact drug by partition chromatography and subsequent determination of the pure isolate. Since the partition coefficient is an extremely sensitive function of molecular structure, it was hoped that this approach will yield the specificity that is lacking in other chemical methods.

The feasibility of the chromatographic technique in isolating the antibiotic is evident from the chromatograms shown in Figs. 3 and 4. These flowing chromatograms were obtained from silicic acid partition columns, with water as the internal phase. The effluent volume corresponding to the maximum concentration of the antibiotic, i. e., the peaks in the diagrams, is a function of the composition of the eluant employed. If a relatively high concentration of ethyl acetate in chloroform is used in the eluant, the peak appears early; conversely, in absence of any ethyl acetate, i. e., with pure chloroform, the drug does not elute out even after 200 ml. of eluant has been consumed. To permit washing out of any materials or components less polar than chloramphenicol from the column before the appearance of the drug in the eluate, it has been found convenient to use about 40 ml. of pure chloroform as the initial eluant. If this is followed by 60 ml. of 10% v/v ethyl acetate in chloroform, all the antibiotic is found in the corresponding eluate.

Since samples to be analyzed may be as aqueous solutions, solids, or dissolved in some organic solvent, a procedure has been developed for each case.

As is readily evident from Fig. 5, the concentration of chloramphenicol in the eluate obtained after partition chromatography may be readily deter-

mined by spectrophotometric analysis over a relatively wide range of wave lengths. This method was therefore employed in analyzing the ethyl acetate–chloroform solutions for antibiotic content. Although the absorption peak in chloroform–ethyl acetate solution is 272 mμ, the samples were analyzed at 278 mμ, the wave length ordinarily used for analyzing aqueous solutions.

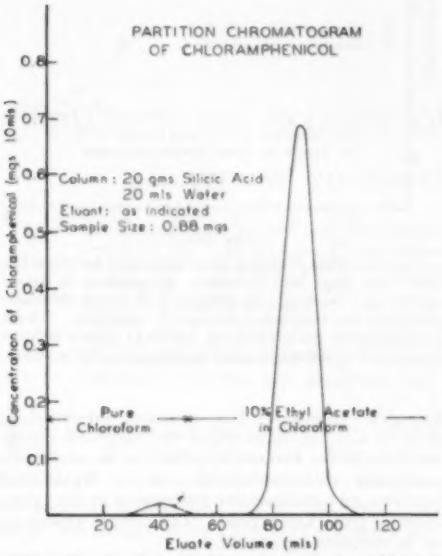


Fig. 3.—Partition chromatogram of chloramphenicol as eluted with 10% (v/v) ethyl acetate in chloroform. The antibiotic, present in a concentration of 440 mcg./ml. was eluted from aqueous solution by the "slurry method" referred to in the text. The first peak may be due to extraneous substances present in the solvent or the system.

THE EXPERIMENTAL METHOD

Reagents.—Ethyl acetate and chloroform, reagent grade. Silicic acid, Mallinckrodt chromatography grade.

Apparatus.—Pyrex glass chromatographic columns approximately 45 cm. long and 2 cm. in diameter. A tight-fitting glass plunger to pack the columns. Beckman Model DU spectrophotometer.

Packing the Columns. The columns are packed according to the procedure given by Higuchi and Patel (7). Distilled water is used as the internal phase and pure chloroform is used as the external phase.

Procedure.—If the chloramphenicol is present in the solid state, it is dissolved in 10% (v/v) ethyl acetate in chloroform. If the chloramphenicol is present in an organic solvent solution, the solvent is removed by evaporation under reduced pressure (*ca.* 25 mm.) and the drug is redissolved in chloroform. The chloramphenicol is then separated as follows.

Exactly 5 ml. of the ethyl acetate–chloroform solution of the antibiotic is pipetted on the surface of the column with care so as to avoid splashing

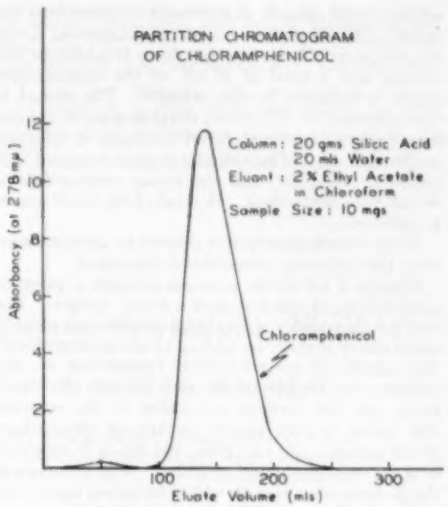


Fig. 4.—Partition chromatogram of chloramphenicol as eluted with 2% (v/v) ethyl acetate in chloroform. The drug, present in a concentration of 2 mg./ml., was added to the column as an ethyl acetate-chloroform solution.

the solution on the sides of the column. A 100-ml. graduated cylinder is used to collect the eluate. After all but the last trace of supernatant liquid has entered the column, 5 ml. of pure chloroform is

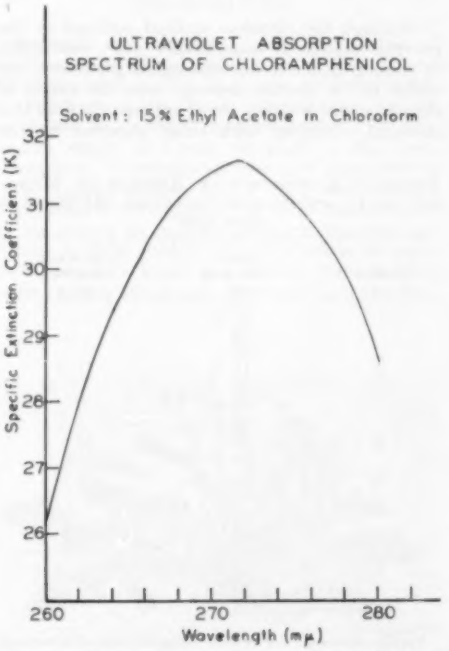


Fig. 5.—Absorption spectrum of chloramphenicol in 15% (v/v) ethyl acetate in chloroform.

added to the column and allowed to drain into the mass. This is repeated with an additional 5 ml. At this point 25 ml. of chloroform is added to the column and a total of 40 ml. of the chloroformic eluate is collected in the cylinder. The eluant is then changed to 10% (v/v) ethyl acetate in chloroform and an additional 10 ml. of eluate is collected in the cylinder. The cylinder is then removed, the contents discarded, and the eluate collected in a 50-ml. volumetric flask. A total of 50 ml. of eluate is collected.

If the chloramphenicol is present in aqueous solution, the following procedure is employed.

Exactly 2 ml. of the aqueous solution is pipetted onto 2 Gm. of silicic acid in a 30-ml. beaker. The two are thoroughly mixed with a nichrome spatula and a slurry is made by adding 15 ml. of chloroform. The slurry is quantitatively transferred to the column, the beaker rinsed with 10 ml. of chloroform, and the rinsings are added to the column. The slurry is then tightly packed on the surface of the column. At this point the eluate is collected in a 100-ml. graduated cylinder. When removing the plunger great care should be exercised to remove any adhering slurry. When almost all of the supernatant liquid has passed into the column, a second slurry prepared from 10 ml. chloroform, 1 Gm. silicic acid, and 1 ml. distilled water is added to the column and tightly pressed onto the mass. After 40 ml. of chloroformic eluate has been collected in the cylinder the same procedure as above is followed.

The contents of the volumetric flasks are analyzed for chloramphenicol content by spectrophotometry.

CORRELATION BETWEEN THE TWO METHODS

Although the chemical method outlined in the previous section is considerably more convenient to employ than the microbiological procedure, the utility of the method depends upon the extent of the agreement between the results obtained by this chemical procedure with those obtained by the biological approach. The evidence presented in Table I and Fig. 6, in which the analytical results obtained by the two methods for a series of samples containing chloramphenicol and its degradation products are compared (8), indicates that the agreement is remarkably good. The results appear to be in complete agreement.

TABLE I.—COMPARISON OF RESULTS OF MICROBIOLOGICAL AND CHROMATOGRAPHIC METHODS OF ANALYSIS[a]

Sample	Microbiological	Chromatographic
1	29	26.5
	25.2	
	29	
	27	
2	17.4	17.4
	15	
	16.4	
	15.5	
3	32.1	31
	29.2	
4	31	31
	35	
	30.8	
5	81	79
	81.5	
6	5.9	6.5
	6.7	
	6.2	

Figures represent per cent of chloramphenicol remaining in degraded samples.

[a] The original concentration of chloramphenicol in the solution subjected to degradation was 0.25%.

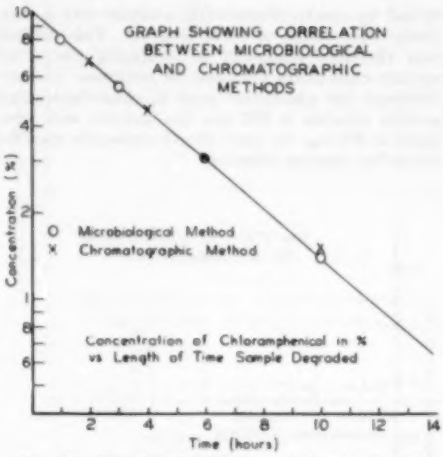

GRAPH SHOWING CORRELATION BETWEEN MICROBIOLOGICAL AND CHROMATOGRAPHIC METHODS
Concentration (%)
O Microbiological Method
X Chromatographic Method
Concentration of Chloramphenicol in % vs Length of Time Sample Degraded
Time (hours)

Fig. 6.—Plot showing the correlation between the microbiological and chemical procedures in determining the residual concentration of intact chloramphenicol in partially degraded samples. These samples were withdrawn at different times from a solution of chloramphenicol undergoing degradation.

The relative precision of the two methods can be seen from the values shown in Tables I and II. In spite of the best technique it is very difficult to obtain precision better than one part in 50 by any biological method because of the many variables involved. With the chromatographic procedure both the accuracy and precision obtainable are directly dependent on the manipulative care employed; good technique readily yields precision of the order of two or three parts per thousand. This is apparent in Table II which shows recovery data on solutions of known composition.

In the procedure described above, smaller concentrations of the antibiotic can be determined by the microbiological method than by the chromatographic procedure. With certain obvious modifications, however, it is apparent that the latter method can be readily adapted so as to allow the detection and estimation of chloramphenicol concentrations of the order of a few micrograms.

The very nature of the chromatographic method prevents it from distinguishing between optical

TABLE II.—RECOVERY OF CHLORAMPHENICOL BY CHROMATOGRAPHIC METHOD

Theoretical, Mg.	Recovered, Mg.	Error, Mg.
4.99	4.99	0.00
4.43	4.39	−0.04
7.40	7.40	0.00
0.645	0.625	−0.02
0.344	0.343	−0.01

isomers. The close agreement obtained by the two methods therefore indicates that racemization is not a significant route of deterioration for chloramphenicol.

REFERENCES

(1) Joslyn, D. A., and Galbraith, M., *J. Bact.*, **59**, 711 (1950).
(2) Kohn, H. I., and Harris, J. S., *J. Pharmacol. Exptl. Therap.*, **77**, 1(1943).
(3) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," Burgess Publishing Co., Minneapolis, 1945.
(4) Bessman, S. P., and Stevens, S. J., *Lab. Clin. Med.*, **35**, 129(1950).
(5) Pepe, T. L., *Bull. chim. farm.*, **88**, 411(1949).
(6) Glazko, A. J., Wolf, L. M., and Dill, W. A., *Arch. Biochem.*, **23**, 411(1949).
(7) Higuchi, T., and Patel, K. P., THIS JOURNAL, **41**, 171 (1952).
(8) Higuchi, T., Marcus, A. D., and Bias, C. D., *ibid.*, **43**, 129(1954).

Antibacterial Activity of *Cassia reticulata* Willd.*

By HEBER W. YOUNGKEN† and ROBERT A. WALSH‡

Extracts of *Cassia reticulata* Willd. inhibit the growth of some microorganisms when tested by the F. D. A. Agar Plate method. A dealcoholized extract, approximately 50 per cent in strength, partially inhibited the growth of *E. coli, A. fecalis, S. lutea, P. vulgaris, S. typhosa, Ps. aeruginosa, M. pyogenes* var. *aureus, M. pyogenes* var. *albus,* and *S. pyogenes* but failed to inhibit the growth of *A. aerogenes, S. marcescens, B. subtilis,* and *H. influenzae.* An aqueous extract was less active. Tests for anthraquinones, emodin, and chrysophanic acid in *C. reticulata* leaflets were positive. The plant and the crude drug, the latter used in the preparation of the extracts for inhibitory activity, are described, and some new characteristics are recorded.

THIS INVESTIGATION was prompted by reports from M. Solera-Viquez of Poas, Costa Rica (1), that patients suffering from arthritis, rheumatoid fever, and some intestinal disorders were relieved by the use of infusions and decoctions of the leaves of *Cassia reticulata* Willd., a tree of the *Leguminosae*, native to South and Central America, and found growing abundantly in certain parts of Costa Rica. Solera-Viquez requested the senior author to carry out a scientific study of the plant to determine its constituents and medicinal properties and supplied an abundance of leaves and specimens of other parts of the plant for the study. This report is a preliminary one and, while mainly concerned with tests of extracts of the leaflets against 14 microorganisms *in vitro*, also includes some additional observations on the plant and crude drug, ascertained in the course of verifying the material used in the tests.

Previously Robbins and his associates (2) had reported that an extract made by boiling dried leaves of *C. reticulata* in water, contained substances that inhibited the growth of *Micrococcus pyogenes* var. *aureus, Mycobacterium phlei,* and *Neisseria gonorrhea,* at concentrations of 84 μg. or less per ml., in *in vitro* tests.

Anchel (3) has found an antibiotic principle in the leaves of *C. reticulata* which she identified as a 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein).

EXPERIMENTAL

Materials and Methods.—The dried leaflets of *Cassia reticulata* Willd. together with other parts of the overground plant were gathered for this study at Palmares, Costa Rica. Identification of the specimens was verified by comparison with herbarium sheets of *Cassia reticulata* in the Gray Herbarium. Antibacterial tests were made with a 40% filtered infusion and a 50% dealcoholized fluidextract of the leaflets. The latter was prepared by macerating the coarsely ground leaflets with one volume of 95% alcohol and two volumes of water for twenty-four hours and filtering through filter paper, adding menstruum gradually until the drug

Fig. 1.—*Cassia reticulata* Willd. Leaf and flowering branch of a tree growing in Costa Rica.

* Received August 21, 1953, from the Massachusetts College of Pharmacy, Boston, Mass.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
† Research Professor of Pharmacognosy and Botany, Massachusetts College of Pharmacy, Boston, Mass.
‡ Research Director, E. L. Patch Co., Stoneham, Mass.

was exhausted, and removing the alcohol by heating the filtrate on a water bath. Tests for anthraquinones, emodin, and chrysophanic acid, following the procedures given in the N. F. IX (4) were positive in both the aqueous and dealcoholized extracts. Both extracts had a *pH* of 5.5.

The method of testing the antibacterial action was the F. D. A. agar plate method (5). The organisms used were taken from twenty-four-hour broth cultures of *Escherichia coli*, *Aerobacter aerogenes*, *Alcaligenes fecalis*, *Sarcina lutea*, *Serratia marcescens*, *Proteus vulgaris*, *Salmonella typhosa*, *Bacillus subtilis*, *Bacillus megatherium*, *Pseudomonas aeruginosa*, *Micrococcus pyogenes* var. *albus*, and *Micrococcus pyogenes* var. *aureus*. Cultures of *Hemophilus influenzae* and *Streptococcus pyogenes* were tested on blood agar plates by streaking the organisms on the plates in gridiron fashion. Sterile cups were then placed on the surface of the agar and filled with the extracts.

After incubation at 37° for twenty-four hours, the plates were examined for inhibition of growth, and the radius of each zone of inhibition measured and recorded. The results of the tests are shown in Table I.

TABLE I.—ACTIVITY OF EXTRACTS OF CASSIA RETICULATA BY F. D. A. AGAR CUP PLATE METHOD AND MODIFICATION[a]

Organism	Radius of Inhibition Zones (mm.)	
	Aqueous Extract	Dealcoholized Extract
E. coli	—	1
A. aerogenes	—	—
A. fecalis	—	2
S. lutea	2	4
S. marcescens	3	—
P. vulgaris	—	4
S. typhosa	—	3
B. subtilis	—	—
B. megatherium	4	7
Ps. aeruginosa	—	3
M. pyogenes var. *aureus*	2	4
M. pyogenes var. *albus*	2	8
H. influenzae	—	—
S. pyogenes	4	4

[a] Modification used in testing *H. influenzae* and *S. pyogenes* (see text for description).

Plant.—*Cassia reticulata* was originally described by Willdenow (6) in his *Enumeratio Plantarum* as follows: "Cassia foliis decemjugis, foliolis oblonga utrinque rotundato-obtusis, subtus pubescenti-canis, extimis majoribus, petiolo eglanduloso, leguminibus compressus." (Cassia with leaves possessing ten leaflets, leaflets oblong, rounded-obtuse at both ends, downy pubescent beneath, the outermost leaflets largest, petiole nonglandular, legume compressed.) He gave its habitat as Para, Brazil.

MacBride (7) described the species growing in Peru as a tall shrub or tree to 15 m. with leaflets in 9 to 12 or 14 pairs, broadly oblong, or slightly obovate and oblique, rounded, or obtuse on both ends, ordinarily 7 to 10 cm. long, 2 to 4 cm. wide, bracts becoming ovate, caducous at anthesis; sepals broad, colored, the larger exceeded by the deeply veined petals; perfect stamens 6, the two larger rostrate, 10 mm. long; pods oblong compressed, lustrous, 1 dm. long or longer, 12 mm. broad or broader.

Fig. 2.—*Cassia reticulata* Willd. Branch bearing two paripinnate leaves and a flowering and fruiting stalk, 1/4×.

The herbarium materials examined in the Gray Herbarium, collected by Sereno Watson in Guatemala and by H. von Wedel in Panama, and the dried leaves, leaflets, and pods received from Costa Rica agreed with these descriptions but, in addition, we found that the leaflets possessed short petiolules, up to 3 mm. in length, their blades up to 15 cm. in length and up to 5 cm. in width with pinnate-reticulate venation. A number of them possessed emarginate to retuse apices. Many had inequilateral bases and all had an entire margin. The flowers were yellow and slightly irregular.

The dried leaflets were olive green and had a subcoriaceous texture, a slightly sweet taste, and a tea-like odor.

REFERENCES

(1) Personal communication.
(2) Robbins, W. J., Kavanagh, F., and Thayer, J. P., *Bull. Torrey Botan. Club*, **74**, 287(1947).
(3) Anchel, M., *J. Biol. Chem.*, **17**, 169(1949).
(4) "The National Formulary," 9th ed., Mack Publishing Co., Easton, Pa., 1950, p. 431.
(5) Rhuehle, G. L. A., Brewer, C. M., U. S. F. D. A. Method of Testing Antiseptics, U. S. D. A. Cir. 198(1938).
(6) Willdenow, D. Car. Lnd., "Enumeratio Plantarum Horti Regii Botanici Berolinensis," Taberna Libraria Scholae Realis, Berlin, 1809, p. 443.
(7) MacBride, J. F., "Flora of Peru," Field Museum of Natural History, Chicago, **13**, No. 3, 1943, pp. 179–180.

Malabar Rauwolfia, *Rauwolfia micrantha* Hook. f.*

By HEBER W. YOUNGKEN†

Rauwolfia micrantha **root has been determined as a substitute for *Rauwolfia serpentina* root in commercial lots offered in increasing amounts to American purchasers. The physical characteristics and histology of the root of *Rauwolfia micrantha* are described. A table of the principal differences between the microscopic structure of the roots of *Rauwolfia micrantha, R. canescens,* and *R. serpentina* is presented. Alkaloidal material is reported for the first time to occur in the root of *Rauwolfia micrantha.* The paper is accompanied by three figures illustrating a leaf and fruiting branch, the histology of a cross section of the root, and the isolated tissue elements of the root of *Rauwolfia micrantha.***

Rauwolfia micrantha Hook. f. (1) is a shrub belonging to the *Apocynaceae*, native to the Malabar district of India. It is characterized by possessing slender branches bearing opposite to 3-whorled, elliptic-lanceolate and oblanceolate, acuminate leaves, few flowered cymes of small tubular flowers and drupe fruits (Fig. 1). It occurs in the Coastal Plain area of India where some lots of commercial roots of *Rauwolfia serpentina* have been gathered.

In another paper (2) the author reported the occasional presence of roots of *Rauwolfia micrantha* in some commercial samples he had examined which were labeled "Rauwolfia Serpentina Root." Since that time he has detected this spurious root to be of more frequent occurrence, and usually found as a complete substitute for genuine Rauwolfia Serpentina drug in an increased number of commercial lots offered to prospective purchasers in the United States.

The purpose of this paper is to report more detailed studies carried out by the author on the roots of Malabar Rauwolfia, including qualitative tests for the presence of alkaloidal material, and some histological distinctions between it, *Rauwolfia serpentina*, and *R. canescens* roots.

Fig. 1.—*Rauwolfia micrantha* Hook. f. Leaf and fruiting branch.

MATERIALS AND METHODS

The materials used consisted of a number of samples of commercial lots labeled "Rauwolfia Serpentina Root" received from importers and manufacturers which were compared with botanically verified roots of *Rauwolfia micrantha* collected for the author by Dr. S. Rajagopalan and his assistant in North and South Travancore, India, and with botanically authenticated roots of *Rauwolfia serpentina* and *R. canescens*, as already described by the author (2). The usual macroscopic, microscopic, and microchemic methods employed in the pharmacognostic examination of crude drugs were used in determining the identity of the spurious commercial Rauwolfia material, later described in this paper. A comparative histological examination was made of temporary and permanent stained mounts of sections of similar diameter, of starch grains in numerous water mounts, and of many slide preparations of teased out tissue elements of pieces of the roots, previously macerated in chromic acid-nitric acid mixture, of *Rauwolfia micrantha, R. canescens,* and *R. serpentina*. Their microscopic structures were recorded.

Qualitative tests for the presence of alkaloidal material in the roots of *Rauwolfia micrantha* were carried out by the use of test tubes containing a filtered alcoholic solution of the ground root into each of which was introduced a different standard alkaloidal reagent, the details of which are later described in this paper. A portion of the precipitate formed with gold chloride T. S., was transferred to a microslide, allowed to crystallize out at room temperature, and the crystals examined microscopically and compared with the crystals formed by allowing several drops of the gold chloride test solution used in the test to crystallize out on a microslide at the same room temperature.

* Received December 30, 1953, from the Massachusetts College of Pharmacy, Boston, Mass.

† Research Professor of Pharmacognosy and Botany, Massachusetts College of Pharmacy, Boston.

RAUWOLFIA MICRANTHA ROOT

Physical Characteristics.—The commercial root occurs in straight, curved, or tortuous segments up to about 10 cm. in length and from 2 mm. to 2.5 cm. in diameter; externally yellowish brown, the bark nearly smooth to irregularly longitudinally striated, to wrinkled and scaly, in thick segments comparatively thin and easily peeling from the wood; fracture of thinner pieces short and irregular, of thicker pieces, irregular and splintery; the thick shorter segments unbreakable by hand; wood pale yellow and very hard in texture; odor indistinct; taste very bitter.

Histology.—The cork shows stratification (Fig. 2, K_1) and consists of 2–7 bands of alternating larger and radiately broader cells with lignified walls and smaller and radiately narrower cells. The larger cork cells in cross sections are up to 75 μ in tangential length and up to 48μ in radial width. The smaller cork cells usually occur in 1–3 or 4 layered bands, the larger cork cells, in 2–9 layered bands. Beneath the cork occurs a phellogen of a layer or two of thin-walled cells followed by the secondary cortex (Co). The latter consists mostly of starch- and crystal-bearing parenchyma with some scattered cells with a yellowish brown amorphous content, comprising up to about 14 layers of tangentially elongated and rounded cells. The subjacent secondary phloem (*P*) consists of a narrow band of starch- and crystal-bearing phloem parenchyma, sieve cells and tubes, and companion cells, and is traversed by phloem rays which curve outward. The phloem rays are 1–4 cells in width. Numerous monoclinic prisms, smaller angular crystals, and a number of twin prisms of calcium oxalate mostly up to 20 μ, occasionally up to 38 μ, occur in the phloem parenchyma. In longitudinal-radial sections, the crystal-cells frequently occurred in rows. Scattered through the phloem are numerous stone cells (*st*), fiber-like stone cells (*scl*), and fibers (Fig. 2). The lignified walls of these are mostly thicker than the lumen. They occur mostly singly but also in groups of up to 24. The fiber-like stone cells were up to 240 μ in length, up to 38 μ in width, and possessed beaded walls. Among them occurred a peculiar diagnostic type with an irregular hooked end. (Fig. 3 (*scl*)) The fibers measured up to 800 μ in length and 38 μ in width.

The xylem (Fig. 2, *X*) consists of a broad zone of numerous wood wedges separated by many wood rays 1–5 cells in width, the walls of all of its elements being lignified. The wood rays as seen in cross section were of two types, one of broader mostly isodiametric cells, the other of narrower, elongated rectangular cells. Both have pitted walls. The wood wedges contain numerous wood fibers, and tracheids with relatively fewer vessels, the latter mostly arranged singly, but occasionally in radiate groups, in pairs, and in nests of up to 7, as viewed in cross sections. The vessel elements have pitted and bordered pitted walls, are circular, oval, or polygonal in cross section with transverse, oblique, and tapered end walls. They are scattered irregularly in the xylem. Perforation plate openings occur in both end and side walls in different vessels. The vessel elements measured up to 450 μ in length and up to 57 μ, rarely up to 72 μ in diameter. A number showed tyloses and those of some roots, gummy

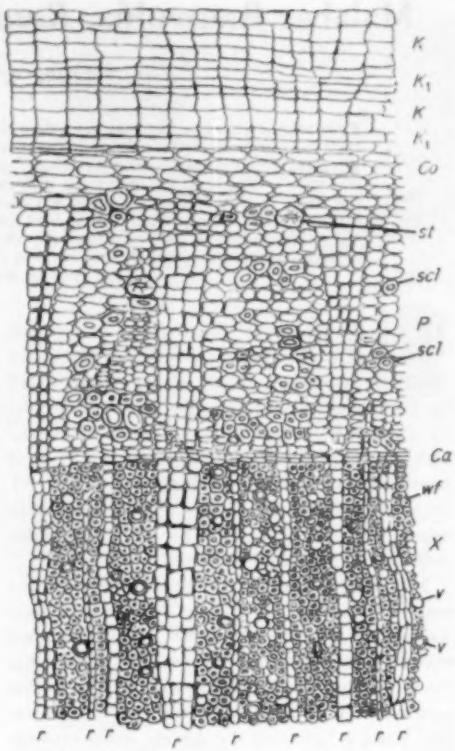


Fig. 2.—*Rauwolfia micrantha* Hook. f. Transverse section of a representative portion of the root. *K*, K_1, stratified cork; *Co*, cortex; *st*, stone cell; *scl*, sclerenchyma; *P*, phloem; *Ca*, cambium; *X*, secondary xylem; *wf*, wood fibers; *v*, vessels; *r*, vascular rays. Magnified.

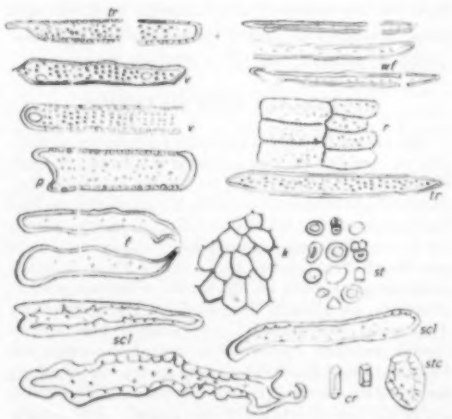


Fig. 3.—*Rauwolfia micrantha* root. Histological elements. *tr*, tracheid; *v*, vessel; *p*, wood parenchyma cell; *wf*, wood fibers; *r*, xylem ray cells; *f*, phloem fibers; *k*, cork tissue in surface view; *st*, starch grains; *scl*, fiber-like stone cells; *cr*, prisms of calcium oxalate; *stc*, stone cell.

TABLE I.—SOME IMPORTANT DISTINCTIONS BETWEEN ROOTS OF RAUWOLFIA MICRANTHA, R. CANESCENS AND R. SERPENTINA[a]

Rauwolfia micrantha	*Rauwolfia canescens*	*Rauwolfia serpentina*
Cork: stratified	Usually not stratified	Stratified
Phloem sclerenchyma: present and more abundant	Present and less abundant	Absent
Vessels: least numerous, mostly up to 57 μ, rarely up to 72 μ in diameter	Most numerous Up to 70 μ, very rarely to 85 μ in diameter	Less numerous Up to 57 μ in diameter
Wood fibers: up to 1460 μ in length	Up to 1500 μ in length, many showing irregular curvatures	Up to 750 μ in length
Stone cells: isodiametric, oval, to fiber-like and without pointed cuticular projections; one form of fiber-like stone cell with hooked end	Isodiametric to fiber-like, irregularly lobed, curved, and polymorphic. One form with septated lumen and pointed cuticular projections	Absent
Starch grains: least abundant, (in powder) the unaltered grains up to 21 μ, the altered, up to 31 μ in diameter	Less abundant, mostly under 15 μ, occasionally up to 26 μ in diameter	Most abundant, the unaltered grains up to 27 μ, the altered, up to 50 μ in diameter
Xylem rays: 1 to 5 cells in width	1 to 5 cells in width	Usually 1 to 5 cells in width, occasionally 1 to 8 cells in width

[a] In 5 transverse sections of different roots of each of these species, all 16 mm. in diameter, the average number of wood wedges and of xylem rays within 1000 μ of the secondary xylem just beneath the cambium was 12 each for *R. micrantha*, 5 each for *R. canescens* and 3.6 each for *R. serpentina*.

lignin deposits. The wood parenchyma cells were more or less rectangular in longitudinal section with lignified and pitted walls, starch contents, and up to 133 μ in length. The wood fibers (Figs. 2, 3, *wf*), representing the most numerous of xylem elements, were up to 1460 μ in length and 38 μ in width. They possessed tapered to abruptly tapered ends, walls with simple and oblique pits which were up to 11 μ in thickness and strongly lignified. The tracheids possessed simple to bordered pits and attained a length of 600 μ. An occasional elongated latex cell with yellowish brown, finely granular contents was found in some longitudinal sections.

Numerous mounts of scraped and ground root were examined in water mounts for starch. The starch grains (Fig. 3, *st*) are simple to 2- or 3-compound, unaltered and altered, the individual grains up to 21 μ and with a central cleft hilum, the altered grains up to 31 μ in diameter; numerous irregular, amorphous masses of brownish color also occurred in the ground root.

Alkaloidal Material.—Qualitative tests for alkaloids were made and reported for the first time on *Rauwolfia micrantha* root. Ten grams of the coarsely ground root were macerated overnight in 100 cc. of 95 per cent alcohol and filtered through chemical filter paper. Portions of this filtrate were poured into each of 5 test tubes. The filtrate fractions in the test tubes were tested by the addition of the following alkaloidal reagents: Eschbach's reagent, Wagner's reagent, gold chloride T. S., Mayer's reagent, and tannic acid T. S. A precipitate occurred in each test tube, indicating the presence of one or more alkaloids. To substantiate further the presence of alkaloidal material in *Rauwolfia micrantha* root, the crystals of a compound formed with the gold chloride as represented by the precipitate formed in the filtered alcoholic solution of the ground root after treatment with gold chloride T. S., were entirely different in structure from the crystals formed by evaporating a thin film of gold chloride on a microslide (see *Methods*).

REFERENCES

(1) Hooker, J. D., "Flora of British India," Vol. III, L. Reeve and Co., London, 1882, p. 663.
(2) Youngken, H. W., THIS JOURNAL, **43**, 144(1954).

VENEREAL DISEASE SYMPOSIUM

The Department of Health, Education, and Welfare has announced the Sixth Annual Symposium on Recent Advances in the Study of Venereal Diseases, to be held in Washington, D. C., April 29 and 30, 1954. The sessions are open to all physicians and workers in allied professions who are interested. Topics to be discussed will cover basic and clinical research, serology, epidemiology, treatment, program operation, and professional education.

A Preliminary Chemical Investigation of *Rauwolfia serpentina**

By WENDELL L. HOLT† and CHRISTOPHER H. COSTELLO‡

The literature of *Rauwolfia serpentina* is summarized and a preliminary investigation of the chemistry of this drug is reported. It was found that of five commercial varieties examined, Dehra Dun and Himalayan materials had the most potent antihypertensive activity, while materials labeled Coastal Plain and Malabar varieties were moderately to grossly adulterated with another species of Rauwolfia. The same types of alkaloids were found in the Dehra Dun and Malabar varieties, but their proportions vary considerably.

Rauwolfia serpentina, Bentham, a drug used as a sovereign remedy in the native medicine of India and Malaya for centuries has recently evinced great interest because of its reported high therapeutic index, and noncritical dosage in the treatment of hypertension. The drug is official in the British *Pharmaceutical Codex* and the *Indian Pharmacopeial List*. Several products containing the whole drug or its alkaloids have recently been introduced.

Despite its potentialities, a review of the literature on Rauwolfia revealed the need of considerably more scientific exploration and documentation, particularly since certain discrepancies exist in the findings of various investigators. This report constitutes a summary of the significant literature of *Rauwolfia serpentina* and a preliminary chemical investigation of several commercial materials in an attempt to compare the nature and proportion of their active principles and to determine optimum methods for their separation.

Five varieties of *Rauwolfia serpentina* roots are reported to be sold on the Indian market according to the place of origin or source of supply. These are Bengal, Bihar, Dehra Dun, Assam, and Ceylon. We have also encountered three other varieties designated as Himalayan, Coastal Plain, and Malabar material. The last two have been found to be moderately to grossly adulterated with an unidentified species of Rauwolfia.

CHEMICAL HISTORY

Chemical investigation was meager until 1931, when the Siddiquis (1) reported the isolation of five alkaloids from the Bihar variety. Three of these alkaloids which they called ajmaline, ajmalinine, and ajmalicine were weakly basic while the other two, serpentine and serpentinine, were strongly basic alkaloids. In 1939, the Siddiquis (2) isolated from the Dehra Dun variety two new alkaloids which they named isoajmaline and neoajmaline respectively.

The alkaloids rauwolfine and isorauwolfine which were isolated by van Itallie and Steenhauer in 1932 (3) were probably identical with ajmaline and isoajmaline, respectively.

In 1944, Gupta, Kahali, and Dutt (4) isolated from the resin of *Rauwolfia serpentina* a nonalkaloidal fraction which had decided hypnotic but no hypotensive properties.

Chatterjee and Bose (5) in 1951 reported the isolation of a new weakly basic alkaloid rauwolfinine from *Rauwolfia serpentina* which had antihypertensive properties.

In 1951, Chakravarty, *et al.* (6), reported on the clinical use of a "new" but unidentified alkaloid called "RS 51" which was purported to be obtained by extraction of the dried alcoholic extract with petroleum ether.

Reserpine, a relatively weakly basic alkaloid possessing sedative activity, was isolated from the oleoresin fraction by Muller, Schlittler, and Bein in 1952 (7). Working independently, Klohs, *et al.* (8), and Neuss, *et al.* (9), isolated reserpine by chromatography of the oleoresin fraction.

The isolation of two more alkaloids from *Rauwolfia serpentina* was reported in 1953. These were weakly basic sarpagin reported by Stoll and Hofmann (10) and raupin, claimed to be isomeric with ajmalinine by Bodendorf and Eder (11).

While little is known about several of the alkaloids reported, some have been characterized and structural formulas proposed, particularly ajmaline (3, 12–16) serpentine (12, 15–18) and reserpine (7–9).

PHARMACOLOGICAL HISTORY

The reports on the pharmacological actions of these alkaloids have been somewhat conflicting. In a series of papers given from 1936 to 1946 Raymond-Hamet (19–23) reported that ajmaline decreased the tone and amplitude of intestinal contractions and produced a strong and lasting fall in blood pressure. Ajmaline had a selective paralyzing action on the vasoconstrictors reactive to adrenalin, acting as a true sympathicolytic. Serpentine was a more potent hypotensive agent with a prolonged

* Received August 21, 1953, from the Research Laboratories of The Columbus Pharmacal Company, Columbus, Ohio.

† Assistant Scientific Director, The Columbus Pharmacal Company, Columbus, Ohio.

‡ Scientific Director, The Columbus Pharmacal Company, Columbus, Ohio.

The authors wish to acknowledge the technical assistance of Mr. Enos E. Vanatta.

action, and inhibiting to peristalsis. Serpentinine neutralized the renal vasoconstrictor action of adrenalin, but did not reduce its hypertensive action. In the absence of adrenalin, however, it had a pronounced depressor and vasodilator action.

Chopra (24–26) and his colleagues found that serpentine produced falls in arterial pressure in both decerebrate and spinal animals, whereas ajmaline and serpentinine raised the pressure in decerebrate animals, but lowered it in spinal animals. Only serpentine was antihypertensive in intact normal cats but all the alkaloids were effective in experimental hypertension, serpentine producing the maximum effect. Serpentine depressed, whereas serpentinine and ajmaline stimulated the intestine. All three were reported as medullary stimulants, not sedatives.

Several investigators have reported the cardiodepressant action of ajmaline, particularly its ability to diminish the transmission of nerve impulses even when the vagi are cut.

Gupta and Kahali (27) found that the total alkaloids separated from Dehra Dun variety of *Rauwolfia serpentina*, depressed only the circulation, whereas total alkaloids of Bihar and Bengal varieties depressed CNS as well. Kapur (28), however, found that total alkaloids of Bihar and Dehra Dun varieties depressed both. Both authorities agree on their hypotensive and respiratory depressant actions but disagree on the actions on uterus and intestine. Kapur believes that because of the wider therapeutic index, the total alkaloidal extracts are safer to use than the individual alkaloids.

Bhatia and Kapur (29) found that both neoajmaline and isoajmaline reduced normal or induced blood pressure in either intact, spinal, or decerebrate animals.

Reserpine, according to Bein (30) shows a very marked hypnotic effect on dogs, cats and rabbits and lowers the blood pressure. He found that reserpine inhibits pressor responses evoked by electrical stimulation of the afferent vagus and sciatic nerves but no inhibition was evoked by electrical stimulation of splanchnic nerves. Stimulation of peristalsis was noted. Reserpine had no peripheral sympathicolytic and ganglionic blocking action but enhanced the action of sympathicomimetic compounds.

According to Achelis and Kroneberg (31), raupin has a more marked adrenolytic action on smooth muscle than the total alkaloids of the drug. It is hypotensive in cats in i. v. doses of 0.5 to 2 mg./Kg. overcoming the pressor effect of adrenalin. The alkaloid was well tolerated by mice in doses of 60 mg./Kg. and was not sedative even at this dosage.

EXPERIMENTAL

Relative Potencies *vs.* Total Alkaloidal Content of Commercial Varieties.—Alcoholic extracts of several samples of the drug, designated by the suppliers as Dehra Dun, Himalayan, Bihar, Coastal Plain, and Malabar materials, respectively, were prepared by percolation with SDA 23A alcohol (10 parts acetone to 90 parts of alcohol) until no further tests for alkaloids with Mayer's reagent were obtained, and compared pharmacologically for hypotensive activity. Detailed results are reported elsewhere (32, 33). It was found that maceration with 2% concentrated ammonia in this solvent prior to percolation materially increased the potency of these extracts. As was expected, these extracts varied widely in potency, not only between different varieties, but also between different samples of the same variety. Generally, Dehra Dun and Himalayan material were the most potent; Coastal Plain and Bihar materials approximately half as potent and Malabar material one-fifth as potent as the Dehra Dun or Himalayan. Since a total of only nine samples were involved it would be premature to assume from these results that any one variety is consistently the most potent until a statistically significant number of different samples were so examined.

The Himalayan, Bihar, Coastal Plain, and Malabar materials were submitted to assay for total alkaloids as prescribed by the *British Pharmaceutical Codex* of 1949 for Rauwolfia to determine the relationship, if any, between total alkaloid content and antihypertensive activity. The supply of Dehra Dun was unfortunately exhausted and could not be run. The percentages of total alkaloids found were as follows: Himalayan 2.20%; Bihar 2.32%; Coastal Plain 1.42%; and Malabar 1.2%.

From this it is quite apparent that the total alkaloid content is not indicative of the antihypertensive potency of the drug, and does not constitute a reliable standard, unless the nature and proportion of the individual alkaloids are known. Until such time as a reliable chemical standard is available, a bioassay based on its hypotensive action should be established for the drug.

It is interesting to note that Dr. Heber Youngken found that the Coastal Plain and Malabar varieties were moderately to grossly adulterated with another species of Rauwolfia,[1] while all the others examined were authentic *Rauwolfia serpentina*. Subsequent chemical examinations revealed considerable differences in the proportion of individual alkaloids between the adulterated and authentic species.

Determination of Optimum Solvent.—In order to determine the best solvent for the extraction of the active principles of the drug from a pharmaceutical standpoint, separate portions of Himalayan sample, the alcoholic extract of which had demonstrated good antihypertensive effect, were extracted with several of the common solvents, and from the resultant extracts the total alkaloids were separated and tested pharmacologically.

In each case the drug was pretreated with stronger ammonia water, and continuously extracted with the solvent to exhaustion in a Soxhlet apparatus.

The total alkaloids were removed from these extracts by a method which differed slightly from the British Pharmaceutical Codex method in these two respects. The alcohol and acetone extracts were made immiscible for acid extraction by adding chloroform to their concentrated solutions, and all extracts were first run through an additional acid extraction to remove the bulk of the impurities.

The results of these experiments are presented in Table I.

Determination of Optimum Method of Total Alkaloid Separation.—Several conventionally used methods of total alkaloid separation as well as those

[1] See Dr. Youngken's article in this issue of THIS JOURNAL, p. 141.

appearing in the literature specifically for Rauwolfia were compared as possible starting points for the isolation and characterization of the individual alkaloids and as assay methods. In each case, *Rauwolfia serpentina* of known antihypertensive activity was used.

The U. S. P. XIV Method of Alkaloidal Drug Assay (Proximate Assays), using extraction method C with ether as the immiscible solvent, yielded a fraction that had little of the drug's antihypertensive activity.

A method recommended by Bakshi (34) was carried out which consisted of extracting the evaporated alcoholic extract with 0.1 *N* HCl, filtering off the resin, precipitating the alkaloids with stronger ammonia, and purifying by repeating the acid extraction and precipitation with ammonia. The resultant total alkaloid fraction was relatively active, but it was found that the resin removed in the process had nearly an equal activity, demonstrating the inefficiency of the acid extraction. Subsequent experience has revealed that ammonia will precipitate the ajmaline group, and the weaker yellow alkaloids, from acid solution, but sodium hydroxide is required to precipitate serpentine.

TABLE I.—COMPARISON OF TOTAL ALKALOIDS AND ANTIHYPERTENSIVE POTENCIES OF EXTRACTS OBTAINED BY VARIOUS SOLVENTS

Solvent	Total Alkaloids, %	Index of Antihypertensive Potency (% drop)
Ethanol	1.54	26
Ether	1.35	19
Acetone	1.86	44
Petroleum ether	.49	15
Chloroform	1.44	33
Benzene	1.39	23
Ethylene dichloride	1.56	29
Direct B. P. C. Method	2.20	7.5

The procedure employed by Chakravarty, *et al.* (6), for the extraction of the alkaloids "RS 51" failed to produce any alkaloidal or antihypertensive material whatsoever in our hands. The method outlined by these investigators consists of extracting the evaporated alcoholic extract with petroleum ether, extracting the petroleum ether extract with 10% HCl, and making the aqueous phase alkaline with ammonia in the presence of chloroform. The alkaloid is supposed to be obtained as a free base on separating and distilling off the chloroform layer.

Because of the structural resemblance between certain Rauwolfia alkaloids and yohimbine, the process commercially employed (35) for the extraction of the latter was tried. This process involves the extraction of the alcoholic extract with CCl_4, the removal of the total alkaloids from the CCl_4 solution with 5% HCl, and subsequent purification. The purification process was not carried out since both the CCl_4 and acid solutions contained negligible amounts of alkaloids as determined by Mayer's reagent, whereas the residue remaining after the extraction process gave strongly positive tests.

The procedure of Schlittler and Schwarz (17) previously mentioned was carried out to the point where the crude ajmaline bases are separated with ethylene dichloride, and the serpentine bases are obtained in the methanol acetic acid fraction.

The potency of these fractions indicates that most of the activity of the drug was recovered quantitatively in these concentrates, with the serpentine fraction having somewhat over twice the potency of the ajmaline fraction.

The method of assay for *Rauwolfia serpentina* in the *British Pharmaceutical Codex* invariably gave the highest yield of total alkaloids by weight. No attempt was made at this time to determine the type and proportion of alkaloids obtained by this method, but the hypotensive activity of the total alkaloids indicates that this method by no means extracts the total hypotensive activity of the drug and is not a reliable standard (see Table I).

The method employed by the Siddiquis (1) was carried to the point where the crude total ajmaline alkaloids and the crude total serpentine alkaloids were separated. The former theoretically contains all the ajmaline, ajmalinine, and ajmalicine and the latter all the serpentinine and serpentine. This scheme of separation, slightly modified from the original method, is outlined in Fig. 1.

When this method was applied to a sample of Dehra Dun variety the alcoholic extract of which had high antihypertensive activity, the combined fractions had only about one-tenth the activity displayed by their equivalent in alcoholic extract of the crude drug.

Since this procedure is somewhat complicated involving numerous extractions and precipitations, much of the activity may have been lost in the process of separation. The resin remaining after this procedure was also found to contain some alkaloids. At any rate, it is apparent that the active principles cannot be separated quantitatively by this method. However, this procedure is extremely valuable as a starting point for the isolation of the various alkaloids, and for approximating the proportion in which the two main groups exist in a given sample. The latter information is highly important since the serpentine group is considerably more potent than the ajmaline group in antihypertensive activity. Consequently a total alkaloid determination may be highly misleading if used as an index of potency unless this proportion is known.

Samples of Dehra Dun and Malabar material were separately submitted to a modification of the method of the Siddiquis (1) for the isolation of their component alkaloids. In both cases the materials were worked up to the crude ajmaline and crude serpentine groups as outlined previously (Fig. 1).

These groups were further separated into crystalline alkaloids and fractions of amorphous material containing individual alkaloids. The same types of alkaloids were found in both varieties but in widely varying proportions. This variation may account for certain differences noted in antihypertensive activity.

SUMMARY

(1) The literature of *Rauwolfia serpentina* is summarized and a preliminary investigation made of its chemistry.

(2) Of the five commercial varieties investigated, Dehra Dun and Himalayan materials had the most potent antihypertensive activity.

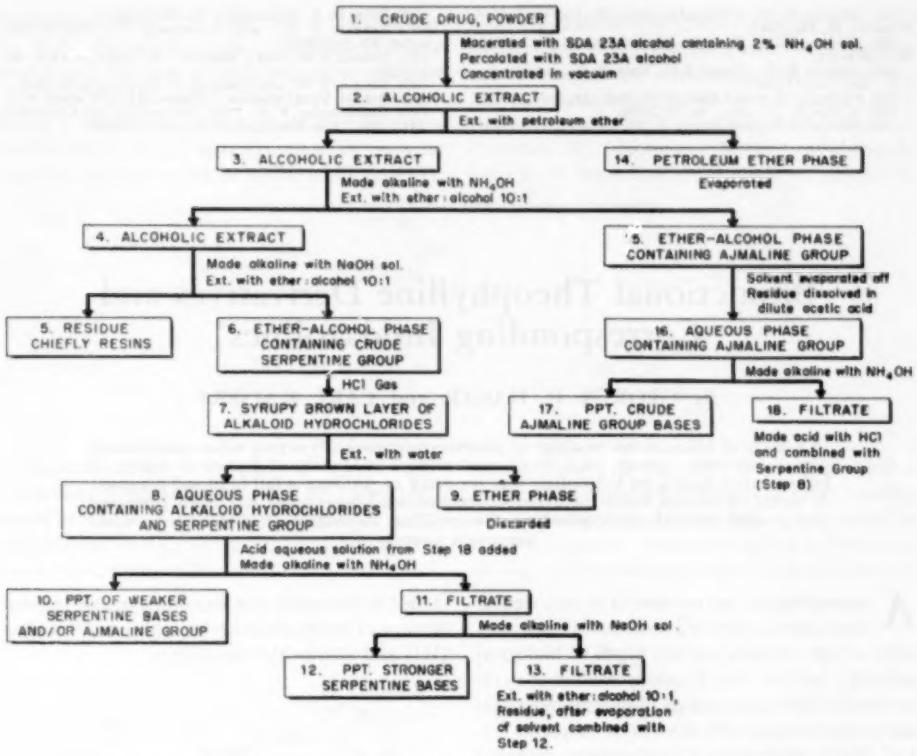

1. CRUDE DRUG, POWDER
Macerated with SDA 23A alcohol containing 2% NH_4OH sol.
Percolated with SDA 23A alcohol
Concentrated in vacuum
2. ALCOHOLIC EXTRACT
Ext. with petroleum ether
3. ALCOHOLIC EXTRACT
14. PETROLEUM ETHER PHASE
Evaporated
Made alkaline with NH_4OH
Ext. with ether : alcohol 10:1
4. ALCOHOLIC EXTRACT
15. ETHER-ALCOHOL PHASE CONTAINING AJMALINE GROUP
Made alkaline with NaOH sol.
Ext. with ether : alcohol 10:1
Solvent evaporated off
Residue dissolved in dilute acetic acid
5. RESIDUE CHIEFLY RESINS
6. ETHER-ALCOHOL PHASE CONTAINING CRUDE SERPENTINE GROUP
16. AQUEOUS PHASE CONTAINING AJMALINE GROUP
Made alkaline with NH_4OH
HCl Gas
7. SYRUPY BROWN LAYER OF ALKALOID HYDROCHLORIDES
17. PPT. CRUDE AJMALINE GROUP BASES
18. FILTRATE
Made acid with HCl and combined with Serpentine Group (Step 8)
Ext. with water
8. AQUEOUS PHASE CONTAINING ALKALOID HYDROCHLORIDES AND SERPENTINE GROUP
9. ETHER PHASE
Discarded
Acid aqueous solution from Step 18 added
Made alkaline with NH_4OH
10. PPT. OF WEAKER SERPENTINE BASES AND/OR AJMALINE GROUP
11. FILTRATE
Made alkaline with NaOH sol.
12. PPT. STRONGER SERPENTINE BASES
13. FILTRATE
Ext. with ether : alcohol 10:1, Residue, after evaporation of solvent combined with Step 12.

Fig. 1.—Method of extraction and separation of Rauwolfia alkaloids into groups.

(3) Materials labeled Coastal Plain and Malabar varieties were moderately to grossly adulterated with another species of Rauwolfia.

(4) The total alkaloid content is not indicative of the antihypertensive activity of the drug and is an unreliable standard unless the proportion of the individual alkaloids is known.

(5) Chloroform, acetone, and ethylene dichloride were found to be the best single solvents for the extraction of the total alkaloids and hypotensive principles from the drug.

(6) A modification of the British Pharmaceutical Codex Assay is suggested as a more accurate standard for the drug.

(7) The method of Siddiqui was found to be most satisfactory for determining the proportion of the main groups of alkaloids and for the isolation of certain of the individual alkaloids.

(8) The same types of alkaloids were found in both Dehra Dun and Malabar varieties, but their proportions vary considerably, which may account for the difference in antihypertensive activity.

REFERENCES

(1) Siddiqui, S., and Siddiqui, R. P., *J. Indian Chem. Soc.*, **8**, 667(1931).
(2) Siddiqui, S., *ibid.*, **16**, 421(1939).
(3) van Itallie, L., and Steenhauer, A. J., *Pharm. Weekblad*, **69**, 334(1932).
(4) Gupta, J. C., Kahali, B. S., and Dutt, A., *Indian J. Med. Research*, **32**, 183(1944).
(5) Chatterjee, A., and Bose, S., *Science and Culture*, **17**, 139(1951).
(6) Chakravarty, N. K., Chaudhuri, M. N., and Chaudhuri, R. N., *Indian Med. Gas.*, **86**, 348(1951).
(7) Muller, J. M., Schlittler, E., and Bein, H. J., *Experientia*, **8**, 338(1952).
(8) Klohs, M. W., Draper, M. D., Keller, F., and Petracek, F. J., *J. Am. Chem. Soc.*, **75**, 4867(1953).
(9) Neuss, N., Boaz, H. E., and Forbes, J. W., *ibid.*, **75**, 4871(1953).
(10) Stoll, A., and Hofmann, A., *Helv. Chim. Acta*, **36**, 1143(1953).
(11) Bodendorf, K., and Eder, H., *Naturw.*, **40**, 342(1953).
(12) Siddiqui, S., and Siddiqui, R. P., *J. Indian Chem. Soc.*, **12**, 37(1935).
(13) Mukherjee, D., Robinson, R., and Schlittler, E., *Experientia*, **5**, 215(1949).
(14) Chatterjee, A., and Bose, S., *ibid.*, **9**, 254(1953).
(15) Siddiqui, S., and Siddiqui, R. P., *J. Indian Chem. Soc.*, **9**, 539(1932).
(16) Raymond-Hamet, M., *Compt. rend.*, **229**, 1165(1949).
(17) Schlittler, E., and Schwarz, H., *Helv. Chim. Acta*, **33**, 1463(1950).
(18) Bader, F., and Schwarz, H., *ibid.*, **35**, 1594(1952).
(19) Raymond-Hamet, M., *Bull. sci. pharmacol.*, **43**, 364(1936).
(20) Raymond-Hamet, M., *Compt. rend. soc. biol.*, **134**, 309(1940).
(21) Raymond-Hamet, M., *ibid.*, **134**, 96(1940).
(22) Raymond-Hamet, M., *Compt. rend.*, **211**, 414(1940).
(23) Raymond-Hamet, M., *ibid.*, **223**, 927(1946).

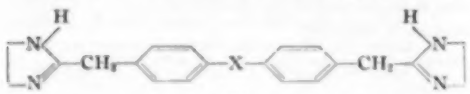
(24) Chopra, R. N., and Chakravarty, M., *Indian J. Med. Research*, **29**, 763(1941).
(25) Chopra, R. N., Bose, B. C., and Gupta, J. C., *ibid.*, **30**, 319(1942).
(26) Chopra, R. N., Gupta, J. C., Bose, B. C., and Chopra, I. C., *ibid.*, **31**, 71(1943).
(27) Gupta, J. C., and Kahali, B. S., *ibid.*, **31**, 215(1943).
(28) Kapur, R. D., *ibid.*, **35**, 57(1948).
(29) Bhatia, B. B., and Kapur, R. D., *ibid.*, **32**, 177(1944).
(30) Bein, H. J., *Experientia*, **9**, 107(1953).
(31) Achelis, J. D., and Kroneberg, T., *Naturwissenschaften*, **40**, 342(1953).
(32) Nelson, J. W., and Schlagel, C. A., THIS JOURNAL, **42**, 324(1953).
(33) Nelson, J. W., and Schlagel, C. A., to be published.
(34) Bakshi, V. M., *Indian J. Pharm.*, **12**, 172(1950).
(35) Hammerslag, F. E., "The Technology and Chemistry of Alkaloids," Van Nostrand, N. Y., 1950, p. 303.

Bifunctional Theophylline Derivatives and Corresponding Imidazolines*

By GEORGE P. HAGER and CARL KAISER†

In a study of bifunctional analogs of pharmacodynamically active substances having one functional group, 4,4′-bis(2-methylene-2-imidazoline)-diphenyl ether, a bifunctional analog of Priscoline was reported to produce a fall in blood pressure. A series of related bis(2-methylene-2-imidazoline)-derivatives has been prepared along with several corresponding theophylline derivatives for testing as vasodepressor agents.

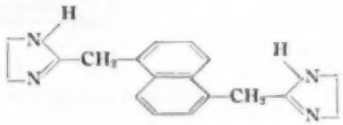
A SYMMETRICAL ARRANGEMENT of two similar functional groups in a molecule is consistent with a high order of certain types of biological activity; and for that reason a number of symmetrical bifunctional analogs of pharmacodynamically active substances have been prepared (1). 4,4′-Bis(2-methylene-2-imidazoline)-diphenyl ether, which bears this relationship to Priscoline, was found to possess some vasodepressor activity (1). Since maximum biological activity in other series of bifunctional compounds is to some degree determined by the intramolecular distance separating the two functional groups, the stem nuclei of a series of bifunctional analogs of Priscoline have been chosen to produce a somewhat regular variation in this distance (I, where X = $-O-$, $-CH_2CH_2-$, $-O(CH_2)_2O-$, and $-O(CH_2)_3O-$). These imidazolines were prepared by



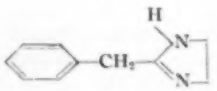
(I)

fusion of the corresponding dinitriles with ethylenediamine *p*-toluenesulfonate by the method described by Oxley and Short (2).

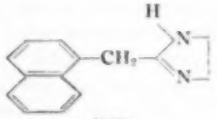
1,5-Bis(2-methylene-2-imidazoline)-naphthalene (II) prepared in the same way was included in the series and was of particular interest because of its structural relationship to Priscoline (III) and also to Privine (IV).



(II)



(III)

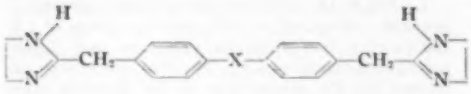


(IV)

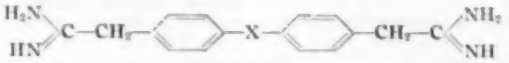
In lowering blood pressure, 1,5-bis(2-methylene-2-imidazoline)-naphthalene appeared to be the most active of the group. However, it was not possible to make a complete evaluation of relative circulatory effects because the compounds inhibited respiration in the doses which appeared to be necessary for the effect on blood pressure. Several of the compounds were spasmogenic when tested using isolated guinea pig intestine. In these tests, 4,4′-bis(2-methyle-e-2-imidazoline)-bibenzyl and 1,5-bis(2-methylene-2-imidazoline)-naphthalene had a spasmolytic effect which was much less pronounced and specific than that of

* Received August 21, 1953, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Maryland, Baltimore, Md.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
† Abstracted from a thesis presented to The Graduate School of the University of Maryland by Carl Kaiser in partial fulfillment of the requirements for the degree of Master of Science.

diphenhydramine. It should be of interest also to study the antibacterial properties of these compounds because of their structural relationship to a parallel series of amidines which were found to be active against *Mycobacterium tuberculosis* $H_{37}Rv$ (3). The imidazolines (V) may be regarded as cyclic analogs of the amidines (VI).

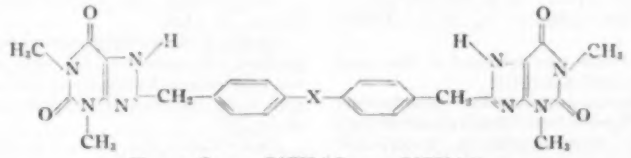


(V)



(VI)

Bifunctional analogs of another type of vasodepressor, 8-benzyltheophylline (4), were prepared from the same intermediates used in the preparation of the bifunctional analogs of Priscoline (see formula VII).



(VII)

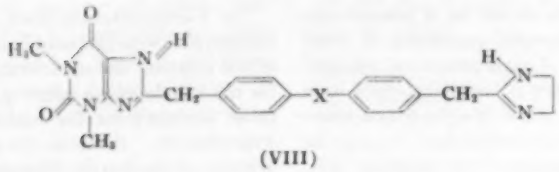
These compounds can be made by fusion of the appropriate dicarboxylic acid with 1,3-dimethyl-5,6-diaminouracil followed by cyclization with the use of sodium hydroxide (4). However, because of the high melting points of the dicarboxylic acids and the tendency of 1,3-dimethyl-5,6-diaminouracil to decompose around 200°, the whole reaction including amide formation and cyclization may be better accomplished by refluxing a solution of the starting materials in phosphorus oxychloride. Although the preparation of 8-phenyltheophylline by fusion of benzoic acid with 1,3-dimethyl-5,6 diaminouracil by the former procedure was unsuccessful (5), this compound was prepared in 39 per cent yield, using phosphorus oxychloride as the solvent.

In preliminary studies, the bifunctional analogs of 8-benzyltheophylline produced an effect on blood pressure which was qualitatively and quantitatively much like that of the monofunctional prototype. The disadvantage of limited water solubility, characteristic of 8-benzyltheophylline, is true to a still higher degree with the bifunctional analogs.

The 8-aralkyltheophyllines such as 8-benzyltheophylline were prepared in an effort to combine in one molecule the structural features necessary for the vasodepressor effect of theophylline with those necessary for the vasodepressor effect of Priscoline (5). However, the pharmacodynamic activity of the 8-aralkyltheophyllines appears to be that which is characteristic of theophylline and the structural determinants of Priscoline-like activity must be presumed lost or suppressed in the combination. Inasmuch as the bifunctional analogs of Priscoline and also those of 8-benzyltheophylline retain to some extent the vasodepressor activity of the parent monofunctional compounds, it seems possible that a hybridization of structures accomplished without modification of the functional groups of either parent substance may combine in one molecule the pharmacodynamic properties of both Priscoline and theophylline. Current studies have as their objective the preparation of a series of such bifunctional analogs of Priscoline and 8-benzyltheophylline (VIII).

EXPERIMENTAL

1,3-Bis[4-(2-methylene-2-imidazoline)-phenoxy]-propane.—A mixture of 6.1 Gm. (0.02 mole) of 1,3-bis(4-cyanomethylphenoxy)-propane (1) and 11.6 Gm. (0.05 mole) of ethylenediamine *p*-toluenesulfonate (2) was heated at 210° for one hour. The product, a salt of the bis(imidazoline) and *p*-toluenesulfonic acid, was dissolved in water and the solution was made alkaline with sodium hydroxide and extracted with chloroform. The chloroform solution was dried using anhydrous sodium sulfate and the chloroform was removed by distillation. The residue was recrystallized from 95% ethanol, and



(VIII)

6 Gm. (76%) of fine, white crystalline 1,3-bis[4-(2-methylene-2-imidazoline)-phenoxy]-propane was obtained; m. p. 198–200° dec.

Anal.—Calcd. for $C_{23}H_{28}N_4O_2$: N, 14.26. Found: N, 14.01.

The dihydrochloride was prepared in the usual way, m. p. 248–250° dec.

1,5-Bis[4-(2-methylene-2-imidazoline)-phenoxy]-pentane.—Prepared in 80% yield from 1,5-bis(4-cyanomethylphenoxy)-pentane (1) by the method described for 1,3-bis[4-(2-methylene-2-imidazoline)-phenoxy]-propane; white crystalline solid, m. p. 164–165° dec.

Anal.—Calcd. for $C_{25}H_{32}N_4O_2$: N, 13.32. Found: N, 13.12.

The dihydrochloride was prepared in the usual way; m. p. 209–210.5°.

4,4′-Bis(2-methylene-2-imidazoline)-bibenzyl.—Prepared in 94% yield from 4,4′-bis(cyanomethyl)-bibenzyl (6); white crystals, m. p. 198–200° dec.

Anal.—Calcd. for $C_{28}H_{34}N_4$: N, 16.17. Found: N, 16.36.

The dihydrochloride was prepared in the usual way; m. p. above 300° dec.

1,5-Bis(2-methylene-2-imidazoline)-naphthalene.—Prepared in 97% yield from 1,5-bis(cyanomethyl)-naphthalene (7); white needles, m. p. 247–248° dec.

Anal.—Calcd. for $C_{18}H_{20}N_4$: N, 19.16. Found: N, 19.15.

4,4′-Bis(8-methylenetheophylline)-diphenyl Ether.—A mixture of 20 Gm. (0.07 mole) of 4,4′-bis(carboxymethyl)-diphenyl ether (8) and 23.8 Gm. (0.14 mole) of 1,3-dimethyl-5,6-diaminouracil was fused at 190° for two hours. The mass solidified partially on heating and was dissolved in 300 cc. of 1% sodium hydroxide by gentle heat. The filtered solution was saturated with carbon dioxide and the precipitated product was recrystallized from dilute acetic acid. A white powder was obtained; m. p. above 300°.

Anal.—Calcd. for $C_{32}H_{32}N_8O_6$: N, 20.21. Found: N, 20.31.

1,3-Bis(4-carboxymethylphenoxy)-propane.—1,3-Bis(4-cyanomethylphenoxy)-propane (1) was hydrolyzed by refluxing in 20% potassium hydroxide in ethylene glycol. After dilution of the reaction mixture with water, the resulting dicarboxylic acid was precipitated by acidifying with hydrochloric acid. It was recrystallized from 95% ethanol using charcoal; result, white crystalline solid, m. p. 216–218°, yield, 70%.

Neutral equivalent.—Calcd. for $C_{19}H_{20}O_6$: 172.2. Found: 178.9.

1,5-Bis(4-carboxymethylphenoxy)-pentane.—Prepared from 1,5-bis(4-cyanomethylphenoxy)-pentane (1) in 76% yield as above described for the corresponding propane derivative; m. p. 184–185°.

Neutral equivalent.—Calcd. for $C_{21}H_{24}O_6$: 186.2. Found: 184.1.

1,3-Bis[4-(8-methylenetheophylline)-phenoxy]-propane.—Prepared in 10% yield by fusion of 1,3-bis(4-carboxymethylphenoxy)-propane and 1,3-dimethyl-5,6-diaminouracil, by the procedure described for the preparation of the diphenyl ether analog. The product was very insoluble in all organic solvents and was purified by dissolving in sodium hydroxide, decolorizing with carbon, and reprecipitating with acetic acid; result, yellow amorphous solid, m. p. above 300°.

Anal.—Calcd. for $C_{31}H_{32}N_8O_6$: N, 18.29. Found: N, 17.80.

The corresponding dipotassium salt was prepared by dissolving a sample of 1,3-bis[4-(8-methylenetheophylline)-phenoxy]-propane in hot 1% ethanolic potassium hydroxide. The product, which crystallized on chilling of the solution, was filtered and washed with ethanol and ether; m. p. above 300°.

Anal.—Calcd. for $C_{31}H_{31}K_1N_8O_6$: N, 16.27. Found: N, 15.83.

1,5-Bis[4-(8-methylenetheophylline)-phenoxy]-pentane.—Prepared in 19% yield by fusion of 1,5-bis(4-carboxymethylphenoxy)-pentane with 1,3-dimethyl-5,6-diaminouracil as described above; m. p. above 300°.

Anal.—Calcd. for $C_{33}H_{36}N_8O_6 \cdot 2H_2O$: N, 16.56. Found: N, 16.34.

In the course of the above preparation it was possible to separate the disodium salt of 1,5-bis[4-(8-methylenetheophylline)-phenoxy]-pentane which was practically insoluble in cold water, from the water-soluble salt of 4-[4-(8-methylenetheophylline)-phenoxypentamethyleneoxy]-phenylacetic acid. The latter was obtained by acidifying the aqueous solution of the salt with acetic acid and recrystallizing the precipitate from glacial acetic acid; m. p. *ca.* 245° dec.

Anal.—Calcd. for $C_{27}H_{30}N_4O_4$: N, 11.06. Found: N, 11.09.

8-Benzyltheophylline. Preparation Using Phosphorus Oxychloride as Solvent.—A mixture of 6.8 Gm. (0.05 mole) of phenylacetic acid, 8.5 Gm. (0.05 mole) of 1,3-dimethyl-5,6-diaminouracil, and 100 cc. of phosphorus oxychloride was refluxed for thirty minutes during which time the mixture became homogeneous. After the excess phosphorus oxychloride had been distilled off under reduced pressure, the residue was mixed with 20 cc. of water. The solid material was purified by dissolving in sodium hydroxide, precipitating with carbon dioxide, and finally recrystallizing from 70% acetic acid; result, fine white needles, m. p. 291°, reported m. p. 289–290° (4), yield, 5.1 Gm. (40%).

8-Phenyltheophylline.—Prepared from benzoic acid in 39% yield by the procedure described for 8-benzyltheophylline; result, yellow crystalline solid, m. p. above 360°. Identified by methylation in the usual way to 8-phenylcaffeine, m. p. 185–186°, reported m. p. 187° (4).

PHARMACOLOGY[1]

A 2% aqueous solution of the dihydrochloride of 1,3-bis{4-(2-methylene-2-imidazoline)-phenoxy}-propane (IX); 1,5-bis{4-(2-methylene-2-imidazoline)-phenoxy}-pentane (X); 4,4′-bis(2-methylene-2-imidazoline)-bibenzyl (XI); or 1,5-bis(2-methylene-2-imidazoline)-naphthalene (XII) was administered intravenously to healthy, nonfasted dogs anesthetized with ether. All four of the compounds in doses of 10 mg./Kg. produced respiratory paralysis. At a 1.0 mg./Kg. dose, X, XI, and XII caused a fall in blood pressure up to 35% of the initial pressure, XII being the most active. At the same time the rate of respiration decreased and stopped completely after five to ten minutes. The compound IX was without effect on blood pressure or respiration at this dose level. No effects were produced by doses of 0.1 mg./Kg.

The imidazolines were also studied for their effect on isolated intestinal strips by the method of Loew and others (9). Guinea pig ileum was suspended in aerated Tyrode's solution and submaximal doses of histamine diphosphate, barium chloride, and acetylcholine bromide were determined. The tissue was treated with a dose of one of the imidazolines or diphenhydramine and, after one minute, the dose of the spasmogenic agent was introduced. Compounds X and XI were found to be spasmogenic while IX and XII were spasmolytic. The latter was especially effective in preventing spasm induced by one of the aforementioned spasmogenic agents, but it was less effective than diphenhydramine and showed little specificity in its action.

4,4′-Bis(8-methylenetheophylline)-diphenyl ether and 1,3-bis{4-(8-methylenetheophylline)-phenoxy}-propane were tested for their effect on the blood pressure of a healthy, nonfasted dog under ether anesthesia. Two per cent aqueous solutions of a mixture of equimolecular amounts of each of these compounds and of sodium acetate, in doses of 0.5 cc./Kg., administered intravenously, produced a 40% decrease in blood pressure in five minutes. In nine minutes the blood pressure returned to 10% below normal and remained there. Respiration was apparently unaffected. A duodenal fistulation was performed on the same dog and 2.5 cc. of the 2% solution per Kg. was administered by way of the gut causing only a slight decrease in blood pressure. Twice this dose produced a lasting 9% decrease.

[1] The authors acknowledge with appreciation the assistance of Dr. C. T. Ichniowski of the Department of Pharmacology, School of Pharmacy, and of Mr. Johnson S. L. Ling of the Department of Pharmacology, School of Medicine, University of Maryland.

SUMMARY

1. A series of bifunctional analogs of Priscoline has been prepared and preliminary pharmacologic studies indicate that the compounds lower blood pressure but only in doses which are inhibitory to the respiration. Activity on isolated guinea pig intestine is also described.

2. Several bifunctional analogs of 8-benzyltheophylline were prepared and found to possess a vasodepressor activity resembling that of the monofunctional prototype.

3. A new procedure for preparing theophylline derivatives substituted in the 8-position is described.

REFERENCES

(1) Hager, G. P., and Liu, W.-C., THIS JOURNAL, **42**, 9 (1953).
(2) Oxley P., and Short, W. F., *J. Chem. Soc.*, **1947**, 497.
(3) Hager, G. P., and Liu, W-C., THIS JOURNAL, **41**, 115 (1952).
(4) Hager, G. P., Krantz, J. C., Jr., and Harmon, J. B., *ibid.*, **42**, 36(1953).
(5) Hager, G. P., Krantz, J. C., Jr., Harmon, J. B., and Burgison, R. M., *ibid.*, **43**, 152(1954).
(6) Reichstein, T., and Oppenauer, R., *Helv. Chim. Acta*, **16**, 1380(1933).
(7) Brunner, A., and Greune, H., U. S. pat. 1,910,462, May 23, 1933; through *Chem. Abstr.*, **37**, 4092(1933).
(8) Tomita, M., and Kimura, K., *J. Pharm. Soc. Japan*, **70**, 44(1950).
(9) Loew, E. R., MacMillan, R., and Kaiser, M. E., *J. Pharmacol. Exptl. Therap.*, **86**, 229(1946).

WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

β-Chlorolactic acid
Pseudocholinesterase
Piperylene
2-Methyl-8-aminoquinoline
Lactic aldehyde
2,3-Dihydrofuran
Laccase
Hedonal
Melibiase
5-Amino-1-pentene
Ethylenesulfonic acid
Dicacodyl
Titanium sulfide
7-Methylxanthine
7-Methylguanine
3,6-Dinitrocarbazole
1,2-Ethanedithiol
Geranic acid
1,3-Dimethyluric acid
Agmantine

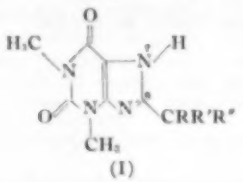
Theophylline Derivatives. II. 8-Aralkyltheophyllines and Related Compounds*

By GEORGE P. HAGER, JOHN C. KRANTZ, Jr., JOHN B. HARMON,† and RAYMOND M. BURGISON

8-Benzyltheophylline, a substance structurally analogous to Priscoline (2-benzyl-2-imidazoline), was reported to be a potent hypotensive agent. A series of related 8-substituted-theophylline derivatives has been prepared for purposes of studying structure-activity relationships and producing vasodepressors which may be still more active, more soluble, and more readily absorbed following oral administration.

THE COMPOUND 8-benzyltheophylline was prepared because of the possibility that hybridization of the structures of theophylline and Priscoline might result in pharmacodynamic properties characteristic of each of these and, therefore, a combination of therapeutic properties desirable in the control of hypertension. This compound appeared to have the site and mechanism of action which are characteristic of theophylline, but it was sufficiently more effective in lowering the blood pressure to justify a further investigation of the class of compounds, 8-aralkyltheophylline derivatives (I), of which 8-benzyltheophylline is one of the simplest members (1).

The use of 8-benzyltheophylline as a drug for treatment of hypertension is seriously limited by its insolubility in water except at a very high *p*H at which the 8-benzyltheophylline anion results from dissociation at position 7 (formula I, where R, R′, and R″ may be hydrogen, alkyl, aryl, substituted alkyl, or substituted aryl).

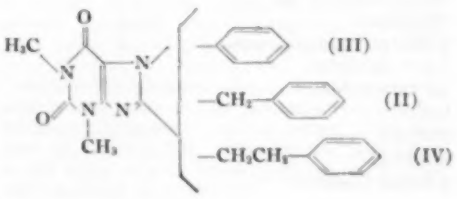


(I)

Unsuccessful efforts to overcome difficulties of absorption which arose from low solubility included the use of a suspension of the sodium salt of 8-benzyltheophylline in oil, prepared by use of a colloid mill, a similar suspension of 8-benzyltheophylline (free acid) in oil, and a suspension of the salt or free acid in mixtures of oil and polysorbate 80.

Since the presence of more strongly polar groups may eventually prove necessary to correct the disadvantage which arises from the lack of solubility in water, certain 8-(substituted-aralkyl) theophyllines were included in the present study. It was found that an electron-withdrawing substituent is detrimental to activity as indicated by the inactivity of 8-(4-sulfobenzyl)-theophylline. Electron-releasing groups similarly located did not decrease the activity appreciably, as indicated by the activity of 8-(4-acetaminobenzyl)-theophylline, but appeared to enhance the activity, as indicated by the properties of 8-(4-aminobenzyl)-theophylline. An acetic acid residue introduced into position 7 of 8-benzyltheophylline in a further attempt at solubilization resulted in loss of most of the activity.

Considerably less active than 8-benzyltheophylline were several 8-aralkyltheophyllines which were homologous with 8-benzyltheophylline; *viz.*, 8-phenyltheophylline and its vinylog, 8-styryltheophylline; 8-(2-phenylethyl)-theophylline and its isostere, 8-(phenoxymethyl)-theophylline. Inasmuch as the 8-benzyltheophylline molecule (II) probably is geometrically intermediate between those of 8-phenyltheophylline (III) and 8-(2-phenylethyl)-theophylline (IV), it is assumed that vasomotor activity of 8-aralkyltheophyllines is not correlated in a fundamental way with molecular shape or size.



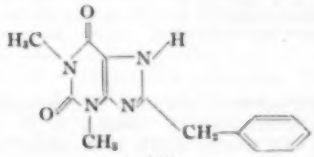
* Received August 21, 1953, from the Department of Pharmaceutical Chemistry, School of Pharmacy and Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md.

Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.

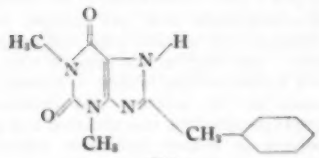
Abstracted in part from a dissertation presented to The Graduate School of the University of Maryland by John B. Harmon in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Present address: E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.

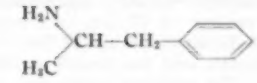
In selecting aralkyl groups which may appear as substituents in the 8-position of theophylline, it proved to be most profitable to make a choice which was based on the chemical properties of other compounds in which the aralkyl groups constituted the stem nuclei. The high rate of nucleophilic substitution of allyl-, benzyl-, and benzhydryl chlorides seems to be paralleled to some extent by a high order of vasomotor activity of 8-allyltheophylline, 8-benzyltheophylline, and 8-benzhydryltheophylline. A similar high order of activity was found in 8-cinnamyltheophylline, a compound which is vinylogous with 8-benzyltheophylline. A ring homolog of 8-benzyltheophylline, 8-(1-naphthylmethyl)-theophylline, was also quite active. The high order of activity possessed by 8-(cyclohexylmethyl)-theophylline (V) appears to be somewhat anomalous and may be another example of the phenomenon responsible for the similar vasomotor activities and similar therapeutic effects of amphetamine (VI) and propylhexedrine (Benzedrex) (VII).



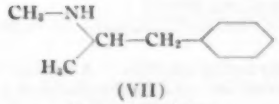
(II)



(V)

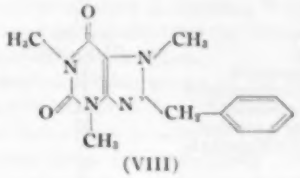


(VI)
Amphetamine



(VII)
Propylhexedrine

The variation of activity with change in structure as above outlined indicates empirically that the 8-aralkyl group exerts its effect on vasomotor properties through its influence on the chemical reactivity of the molecule. Because of the low order of activity of 8-benzylcaffeine (VIII) and 8-benzyltheophylline-7-acetic acid, it appears possible that the 8-aralkyl group present in an 8-aralkyltheophylline which is unsubstituted at position 7 may influence biological activity by its effect on the dissociation of the molecule at position 7 or by its effect on subsequent reactions of the anion formed by such a dissociation (Fig. 1).[1]



(VIII)

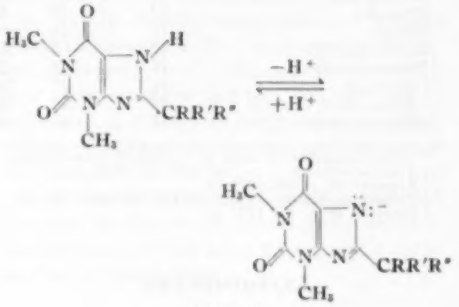


Figure 1

In Table I, a number of the 8-aralkyltheophyllines which have been prepared and tested for vasomotor activity have been listed together with pertinent data.[2]

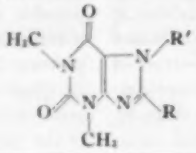
The 8-aralkyltheophyllines were for the most part prepared by fusion of a mixture of 1,3-dimethyl-5,6-diaminouracil and the appropriate carboxylic acid to produce an amide which was then cyclized by dissolving in sodium hydroxide solution (2). When ring closure could not be effected by sodium hydroxide, phosphorous oxychloride, used as directed by Elion and others (3), produced the desired results. The amide intermediate in the preparation of 8-phenyltheophylline was obtained by a Schotten-Baumann acylation of 1,3-dimethyl-5,6-diaminouracil using benzoyl chloride. When the reaction mixture was warmed, the precipitated amide dissolved and formed 8-phenyltheophylline. This compound was previously prepared by a cyclodehydrogenation of the benzylidene derivative of 1,3-dimethyl-5,6-diaminouracil (4).

---

[1] 8-Benzyltheobromine, m. p. 225–226° (*Anal.*—Calcd. for $C_{14}H_{14}N_4O_2 \cdot H_2O$: N, 19.44. Found: N, 19.65), bears a methyl substituent at position 7 and is also inactive.

[2] The results of pharmacologic studies carried out by Dr. John C. Krantz, Jr., and further studies which are still in progress, will be reported in detail in a later publication.

TABLE I.—8-ARALKYLTHEOPHYLLINES AND RELATED COMPOUNDS



R	R′	Yield,[a] %	M. P., °C.	Formula	Analysis, % N— Calcd.	Found
Benzyl-	H	80	289–290[b]	....	....	....
Benzyl-	—CH_3	93	158–159[d]	....	....	....
Benzyl-	—CH_2COOH	28	222.5–223	$C_{14}H_{14}N_4O_4 \cdot H_2O$[e]	16.18	15.59
4-Sulfobenzyl-	H	14	Above 360	$C_{14}H_{14}N_4O_5S \cdot H_2O$	15.21	15.32
4-Acetaminobenzyl-	H	53	294–300 dec.	$C_{16}H_{17}N_5O_3$	21.40	21.77
4-Aminobenzyl-[h]	H	40	297–298 dec.	$C_{14}H_{15}N_5O_2$	24.55	24.03
Cyclohexylmethyl-	H	44	234.8–235	$C_{14}H_{20}N_4O_2$	20.28	19.98
1-Naphthylmethyl-	H	41	303–306 dec.	$C_{18}H_{16}N_4O_2 \cdot H_2O$	16.56	16.20
Allyl-	H	29	273–273.5[g]	$C_{10}H_{12}N_4O_2 \cdot H_2O$	23.52	23.33
Benzhydryl-	H	43	282.5–283	$C_{20}H_{18}N_4O_2$	16.18	16.19
Phenyl-[f]	H	40	Above 360[g]	....	....	....
Styryl-	H	18	Above 360	$C_{15}H_{14}N_4O_2$	19.85	19.91
2-Phenylethyl-	H	60	259–260	$C_{15}H_{16}N_4O_2$	19.71	20.25
Phenoxymethyl-	H	41	224–225	$C_{14}H_{14}N_4O_3$	19.57	19.56
Cinnamyl-	H	65	242.5–243.5	$C_{16}H_{16}N_4O_2$	18.91	18.58
4-Chlorobenzyl-	H	17	295–297 dec.	$C_{14}H_{13}ClN_4O_2$	18.38	18.09
4-Bromobenzyl-	H	40	292	$C_{14}H_{13}BrN_4O_2$	16.04	15.85

[a] Yields calculated on basis of 1,3-dimethyl-5,6-diaminouracil except where R′ is —CH_2COOH or —CH_3.
[b] Reported m. p. 286–287° (1).
[c] Neutral equivalent. Calcd. 328.3. Found: 321.8.
[d] Reported m. p. 161–163° (5).
[e] Sublimes at 215°.
[f] Identified by conversion to 8-phenylcaffeine, m. p. 184°. Reported m. p. 187° (5).
[g] Sublimes at 250°.
[h] Prepared by Carl Kaiser.

EXPERIMENTAL

General Procedure for Preparation of 8-Aralkyltheophyllines.—Unless otherwise indicated in the following, the compounds described in Table I were prepared by the general procedure.

A mixture, made by triturating 1,3-dimethyl-5,6-diaminouracil with 1–1.25 *M* equivalent of the appropriate carboxylic acid, was heated in an open wide-mouth flask or beaker at 120–200° in an oil bath. The mixture usually softened or fused and then resolidified after one to four hours at the elevated temperature. The solid intermediate amide formed in this way was broken up and sometimes powdered, and then heated with 5–10% sodium hydroxide until solution was complete. The alkaline solution was treated with decolorizing carbon and then saturated with carbon dioxide. The 8-aralkyltheophylline which precipitated was recrystallized from water, alcohol, glacial or dilute acetic acid.

In the preparation of 8-(phenoxymethyl)-theophylline, the cyclization in sodium hydroxide solution was accomplished as rapidly as possible because of the alkaline fission of the phenoxy group which was observed when heating was prolonged.

8-Benzyltheophylline-7-acetic Acid.—A mixture of 10 Gm. (0.037 mole) of 8-benzyltheophylline and 4 Gm. (0.042 mole) of monochloroacetic acid in 100 cc. of water was neutralized with 45 cc. of 2 *N* sodium hydroxide and refluxed for three hours. Unreacted 8-benzyltheophylline was precipitated by passing carbon dioxide into the solution. After filtration, 8-benzyltheophylline-7-acetic acid was precipitated by acidification with hydrochloric acid and purified by recrystallization from alcohol.

8-Benzylcaffeine.—Prepared by methylation of 8-benzyltheophylline by treating it in alkaline medium with methyl sulfate in the usual way.

8-(4-Sulfobenzyl)-theophylline.—An aqueous solution of 9.5 Gm. (0.056 mole) of the sodium salt of 4-sulfophenylacetic acid was acidified with 1.6 cc. of concentrated sulfuric acid and refluxed for two hours. The solution was treated with 6 Gm. of sodium hydroxide and heated to boiling. After acidification of the solution and chilling, 8-(4-sulfobenzyl)-theophylline was obtained and purified by dissolving in sodium hydroxide solution and precipitating with hydrochloric acid.

1,3-Dimethyl-6-amino-5-diphenylacetylaminouracil.—A mixture of 17 Gm. (0.1 mole) of 1,3-dimethyl-5,6-diaminouracil and 22 Gm. (0.105 mole) of diphenylacetic acid was heated at 120–125° for two hours. The resulting amide was purified by decolorizing a solution of the crude product in glacial acetic acid with the use of decolorizing carbon, and diluting with water. Yield: 28 Gm. (78%), m. p. 285–286°.

Anal.—Calcd. for $C_{20}H_{20}N_4O_3$: N, 15.38. Found: 14.94.

8-Benzhydryltheophylline.—A mixture of 27 Gm. (0.074 mole) of 1,3-dimethyl-6-amino-5-diphenylacetylaminouracil and 250 cc. of phosphorous oxychloride was refluxed for four hours. The excess phosphorous oxychloride was distilled off under reduced pressure and the tarry residue was treated with ice, made strongly alkaline with 25% sodium hydroxide and warmed until dissolved. 8-Benzhydryltheophylline was precipitated by carbon dioxide and purified by dissolving in alkali, decolorizing with carbon, and reprecipitating with carbon dioxide.

8-Phenyltheophylline.—To a cooled solution of 5 Gm. (0.03 mole) of 1,3-dimethyl-5,6-diaminouracil in 100 cc. of 10% sodium hydroxide, 4.85 Gm. (0.035 mole) of benzoyl chloride was added. After shaking the mixture thoroughly, it was warmed to dissolve the precipitated white solid, and 8-phenyltheophylline was precipitated by carbon dioxide. The product was recrystallized from glacial acetic acid, and identified by methylation to 8-phenylcaffeine.

8-Styryltheophylline.—A mixture of 17 Gm. (0.1 mole) of 1,3-dimethyl-5,6-diaminouracil and 15 Gm. (0.101 mole) of cinnamic acid was heated at 120° for two hours. The crude amide obtained in this way was refluxed for five hours with 250 cc. of phosphorous oxychloride. The excess phosphorous oxychloride was removed by distillation under reduced pressure and the residue was treated with ice and made alkaline with sodium hydroxide. Treatment of the solution with carbon dioxide precipitated 8-styryltheophylline. The product was a light yellow solid.

PHARMACOLOGY

In general, the 8-aralkyltheophyllines were effective in producing a fall of blood pressure when injected intravenously. This depressor response did not prevail upon oral administration owing to the lack of absorption of these compounds from the gastrointestinal tract. Intravenously, depressor responses were obtained varying from 10 to 30% by doses of 6–13 mg./Kg. Atropine, Etamon, Priscoline, and decerebration did not modify the depressor response to these compounds. By poisoning the capillary bed with sodium arsenate the depressor response was likewise not obliterated. It is therefore concluded that the action of these drugs is directly on the arterial musculature. The pattern of the electrocardiogram was not significantly altered by the injections of these theophylline derivatives. These compounds, which were tested for toxicity, had LD_{50}'s in the rat upon intraperitoneal injection of 200 to 400 mg./Kg. This places them in the same category of toxicity as aminophylline.

SUMMARY

1. A series of 8-aralkyltheophyllines related to 8-benzyltheophylline has been prepared in a search for a theophylline derivative as effective as 8-benzyltheophylline in lowering blood pressure but more manageable from the standpoint of administration and absorption.

2. Polar solubilizing groups of the electron-releasing type located in the aralkyl group appear not to be detrimental to activity. An electron-withdrawing group (the sulfo group) similarly located, appears to decrease activity greatly.

3. In the 8-aralkyltheophyllines it appears that the adjacent position 7 should be free to permit dissociation and formation of an anion. Solubilizing groups as in 8-benzyltheophylline-7-acetic acid, and nonpolar groups, as in 8-benzylcaffeine or 8-benzyltheobromine, are detrimental to activity.

4. Groups which are electronically constituted in a way similar to the benzyl group enhance vasodepressor activity when substituted in the 8-position of theophylline.

REFERENCES

(1) Hager, G. P., Krantz, J. C., Jr., and Harmon, J. B., THIS JOURNAL, **42**, 36(1953).
(2) Traube, W., *Ber.*, **33**, 3052(1900).
(3) Elion, G. B., Burgi, E., and Hitchings, G. H., *J. Am. Chem. Soc.*, **73**, 5235(1951).
(4) Traube, W., and Nithack, W., *Ber.*, **39**, 227(1906).
(5) Cook, A. H., and Thomas, G. H., *J. Chem. Soc.*, **1950**, 1884.

1954 Symposium on Antibiotics

Preliminary Announcement

A SYMPOSIUM ON ANTIBIOTICS will be held on October 20, 21, and 22, 1954, in Washington, D. C., under the auspices of the Department of Health, Education, and Welfare in cooperation with the journal *Antibiotics and Chemotherapy*. The symposium will follow the general plan of the successful 1953 conference, the transactions of which have just been made available in a 600-page clothbound book.

Those interested in presenting papers are invited to submit proposed titles and abstracts for the program.

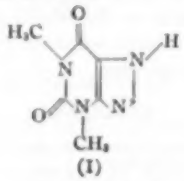
A further announcement giving a definite date for submission of abstracts will be issued.

Theophylline Derivatives. III. 8-(9-Fluorenyl)-Theophylline and Related Compounds*

By GEORGE P. HAGER, CASIMIR T. ICHNIOWSKI, and BERNARD MISEK

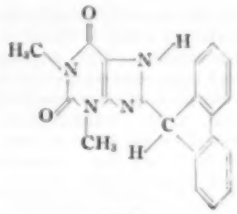
A further investigation of 8-aralkyltheophyllines was undertaken following the observation that maximum depressor activity was found in those compounds in which the 8-aralkyl group was a benzyl group, the related benzhydryl group, or the vinylogous cinnamyl group. A comparison of the vasodepressor effects of 8-benzyl-, 8-benzhydryl-, 8-(2-thenyl)-, 8-(3-thenyl)- and 8-(9-fluorenyl)-theophyllines indicated a pharmacodynamic homogeneity as regards the first four. The 8-(9-fluorenyl)-theophylline produced qualitatively different responses in blood pressure experiments. The 9-fluorenyl- and 9-fluorenylidenemethyl- analogs of 2-benzyl-2-imidazoline (Priscoline) were also prepared for comparison of pharmacodynamic properties.

SUBSTITUTION of the 8-position of the theophylline molecule (I) with a benzyl group (1) or with an aralkyl group constituted electronically like the benzyl group including the benzhydryl and the vinylogous cinnamyl groups (2), resulted in an increase in the vasodepressor properties characteristic of theophylline.

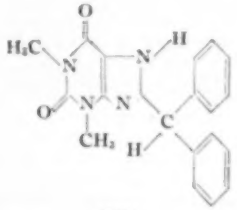


(I)

The insolubility of the 8-aralkyltheophyllines in water and the resulting irregular absorption following oral administration made apparent that a strongly polar solubilizing group must be present. Preliminary experiments indicated that electron-releasing groups in the 8-aralkyl-substituent are not detrimental to activity (2). In connection with a study of a series of theophylline derivatives that had such solubilizing polar substituents, several compounds were prepared and tested to provide a basis for the selection of an aralkyl group for use in a series of 8-(substituted-aralkyl)-theophyllines. For this purpose, the isosteric 8(2-thenyl)-theophylline and 8-(3-thenyl)-theophylline were prepared for comparison with 8-benzyltheophylline; and 8-(9-fluorenyl)-theophylline (II) was prepared for comparison with 8-benzyhydryltheophylline (III). The structures of the latter two compounds are related in the same way as those of Trasentine and Pavatrine, a relationship which has been termed "bioisosteric" (3).



(II)



(III)

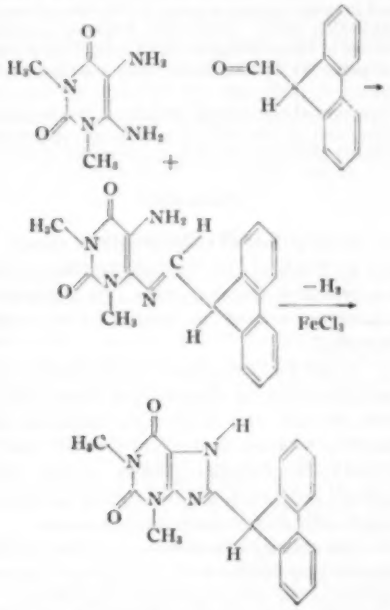
The antispasmodic fluorene-9-carboxylate (Pavatrine) has a greater activity but the same toxicity as the diphenylacetate (Trasentine) (4). Furthermore, the fluorenyl group appears to exhibit, to a greater extent, the tautomeric electronic polarization of the molecular system which is characteristic of the benzyl and benzhydryl groups—a polarization which appears to be correlated with the vasodepressor activity of 8-aralkyltheophyllines (2) just as it may be correlated with certain chemical properties of toluene, diphenylmethane, and fluorene (acid dissociation) (5, 6) or with those of their derivatives or substitution products. For these reasons it was hoped that 8-(9-fluorenyl)-theophylline would prove to be more active than the 8-aralkyltheophyllines previously tested.

* Received August 21, 1953, from the Department of Pharmaceutical Chemistry and Department of Pharmacology, School of Pharmacy, University of Maryland, Baltimore, Md.

Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.

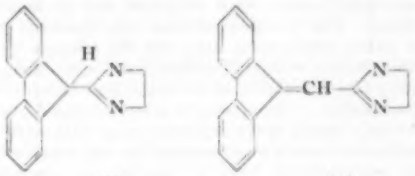
Abstracted in part from a thesis presented to The Graduate School, University of Maryland, by Bernard Misek in partial fulfillment of the requirements for the degree of Master of Science.

The 8-(2-thenyl)-theophylline and 8-(3-thenyl)-theophylline were prepared from 2-thienylacetic acid and 3-thienylacetic acid, respectively, by the procedure generally employed for 8-aralkyltheophyllines, *viz.*, fusion of the carboxylic acid with a little less than a mole equivalent of 1,3-dimethyl-5,6-diaminouracil and cyclization of the intermediate amide by use of sodium hydroxide (1). The 8-(9-fluorenyl)-theophylline could not be prepared by this procedure because of decarboxylation of fluorene-9-carboxylic acid at the high temperature necessary for fusion. The compound was prepared by a cyclodehydrogenation of the Schiff base formed by reaction of 9-formylfluorene with 1,3-dimethyl-5,6-diaminouracil by application of a procedure described by Traube (7).



In blood pressure–respiration experiments on anesthetized dogs, 8-benzyl-, 8-benzhydryl-, 8-(2-thenyl)- and 8-(3-thenyl)-theophyllines were found to have qualitatively and quantitatively similar vasodepressor effects, with the two thenyl derivatives being somewhat less active than the other two. A fall in blood pressure without a concomitant effect on respiration was produced by moderate doses of all four compounds. The effects of 8-(9-fluorenyl)-theophylline differed qualitatively from those of the other 8-aralkyltheophyllines and consisted of a pronounced transient fall in blood pressure with a simultaneous transient cessation of respiration, followed by a longer compensatory rise in blood pressure and respiratory recovery. Subsequent to the acute depressor-pressor phase, there ensued a gradual decrease in blood pressure which seemed to stabilize at a level lower than that prior to the administration of the substance. On the basis of these results, further improvements in the pharmacodynamic properties of compounds with the activity characteristic of 8-benzyltheophylline may possibly be achieved by modification through substitution of 8-benzyl- or 8-benzhydryltheophyllines.

Inasmuch as 8-benzyltheophylline may be regarded as a hybrid of theophylline and Priscoline in which the 2-imidazoline group of the latter has been replaced by theophylline, 2-(9-fluorenyl)-2-imidazoline (IV) and 2-(9-fluorenylidenemethyl)-2-imidazoline (V) were also prepared either by reaction of the corresponding nitrile with ethylenediamine *p*-toluenesulfonate (8) or by the reaction of the ethyl ester of the corresponding carboxylic acid with ethylenediamine (9). Preliminary pharmacologic testing of the substances produced variable results. However, in general they appear to exert a vasodepressor effect accompanied by a transitory inhibition of respiration.



(IV) (V)

EXPERIMENTAL

The preparation of 8-benzyltheophylline and 8-benzhydryltheophylline is reported elsewhere (1, 2).

8-(2-Thenyl)-theophylline.—A mixture of 5 Gm. (0.035 mole) of 2-thienylacetic acid[1] and 4.8 Gm. (0.028 mole) of 1,3-dimethyl-5,6-diaminouracil was heated in an open flask at 120° for about fifteen minutes during which time the melt solidified. The solid intermediate amide was dissolved by heating in 10% sodium hydroxide. The solution was decolorized with carbon and saturated with carbon dioxide. The precipitated theophylline derivative was recrystallized from ethanol or a mixture of equal parts of ethanol and glacial acetic acid and finally washed with ether; result, long white needles, m. p. 268–270° dec., 66% yield.

Anal.—Calcd. for $C_{13}H_{12}N_4O_2S$: N, 20.28. Found: N, 20.10.

8-(3-Thenyl)-theophylline.—Prepared in 60% yield from 3-thienylacetic acid[2] as described for the 2-thenyl isomer; result, long white needles, m. p. 277–278° dec.

[1] Prepared by the hydrolysis (10) of 2-thienylacetonitrile supplied through the courtesy of Dr. E. Campaigne.
[2] Prepared by the hydrolysis (11) of 3-thienylacetonitrile supplied through the courtesy of Dr. E. Campaigne.

Anal.—Calcd. for $C_{12}H_{12}N_4O_2S$: N, 20.28. Found: N, 20.28.

8-(9-Fluorenyl)-theophylline.—A solution of 25 Gm. (0.129 mole) of 9-formylfluorene (12) and 22 Gm. (0.129 mole) of 1,3-dimethyl-5,6-diaminouracil in sufficient absolute ethanol was refluxed until a solid mass precipitated. A solution of 42.5 Gm. (0.258 mole) of anhydrous ferric chloride in absolute ethanol was then added slowly with stirring, and the mixture was refluxed until all solid material dissolved. Most of the ethanol was distilled off and the residual syrupy liquid was mixed with water. The precipitated 8-(9-fluorenyl)-theophylline was dissolved in 2% sodium hydroxide, precipitated by saturation of the alkaline solution with carbon dioxide, and recrystallized from toluene; result, white crystals, m. p. 273–274° dec., 20% yield.

Anal.—Calcd. for $C_{20}H_{16}N_4O_2 \cdot H_2O$: N, 15.43. Found: N, 15.40.

The compound turned pink on standing exposed to the atmosphere and the pink color was again lost on recrystallization from toluene. The pink material had the same melting point (273–274° dec.) and analytical values indicated that the color resulted concurrently with the formation of a higher hydrate.

Anal.—Calcd. for $C_{20}H_{16}N_4O_2 \cdot 4H_2O$: N, 13.47. Found: N, 13.34.

2-(9-Fluorenyl)-2-imidazoline.—A mixture of 3.82 Gm. (0.02 mole) of 9-cyanofluorene (13) and 4.64 Gm. (0.02 mole) of ethylenediamine *p*-toluenesulfonate was heated under reflux at 190–200° for about one hour or until ammonia was no longer evolved. The reaction mixture was dissolved in hot dilute hydrochloric acid and the solution was made alkaline with 10% sodium hydroxide, precipitating 4.25 Gm. (90%) of the crude 2-(9-fluorenyl)-2-imidazoline. The product was recrystallized from benzene; result, white crystals, m. p. 178.5–180°. The hydrochloride was prepared in the usual way and recrystallized from a mixture of absolute ethanol and ether, m. p. 234–235° dec.

Anal.—Calcd. for $C_{16}H_{14}N_2Cl$: N, 10.35. Found: N, 10.11.

2-(9-Fluorenylidenemethyl)-2-imidazoline.—A mixture of 18 Gm. (0.072 mole) of ethyl dibenzofulvenecarboxylate (14) and 34 Gm. of ethylenediamine was heated in a pressure bottle for thirty-six hours on a steam bath. The excess ethylenediamine was removed by distillation under reduced pressure and the residue was recrystallized from absolute ethanol; result, white crystals, m. p. 224–225° dec., 8.8 Gm. (50%).

Anal.—Calcd. for $C_{17}H_{14}N_2 \cdot 2H_2O$: N, 9.92. Found: N, 9.81.

The hydrochloride was prepared in the usual way, m. p. 207–210° dec.

PHARMACOLOGY

Solutions of 2.5 to 10 mg. per cc. of the theophylline derivatives were prepared by dissolving them in 0.1 *N* sodium hydroxide and neutralizing partially to *p*H 10–11 with 0.1 *N* hydrochloric acid. To increase the stability of the solutions, ethanol was added to a concentration of 10%. Such solutions were prepared fresh and were stable for several hours with the exception of the solution of 8-(9-fluorenyl)-theophylline which precipitated within one hour.

The solutions were injected intravenously (femoral vein) into dogs anesthetized by intraperitoneal administration of Dial with Urethane (Ciba). Carotid blood pressure was recorded with a mercury manometer while respiration was recorded with a Marey tambour attached to a tracheal cannula. Comparison of the five theophylline derivatives was made at a dose level of 2.5 mg. per Kg. With the exception of the 8-(9-fluorenyl)-theophylline, the several 8-aralkyltheophyllines tested lowered the blood pressure 20–30% of that of the preinjection level. Recovery to approximately control blood pressure levels occurred usually within three minutes. Respiration appeared to be unaffected.

The 8-(9-fluorenyl)-theophylline produced an acute depressor response—40–90-mm. drops from levels of 115 and 126 mm. with a simultaneous cessation of respiration being observed. Both of these effects were transient, lasting fifteen to thirty seconds. In the next three to four minutes, the blood pressure rose to a point 30–60 mm. above the preinjection level. Thereafter followed a gradual decrease in blood pressure which seemed to stabilize at a point 30–40 mm. lower than the preinjection level.

Injection of the solvent vehicle at the beginning, during, and at the end of a series of comparisons produced either no effect or a rise of 10–15 mm.

SUMMARY

1. Several 8-aralkyltheophyllines closely related to 8-benzyl- or 8-benzhydryltheophylline were prepared together with two 2-aralkyl-2-imidazolines related to 2-benzyl-2-imidazoline (Priscoline).

2. A careful comparison of the pharmacodynamic effects of the theophylline derivatives on blood pressure and respiration indicated that 8-benzyl-, 8-benzhydryl-, 8-(2-thenyl)- and 8-(3-thenyl)-theophyllines possess similar pharmacologic properties, the two former compounds being slightly more active vasodepressors.

3. The activity of 8-(9-fluorenyl)-theophylline was more pronounced and qualitatively different from that of the other members of the series.

REFERENCES

(1) Hager, G. P., Krantz, J. C., Jr., and Harmon, J. B., THIS JOURNAL, **42**, 36(1953).
(2) Hager, G. P., Krantz, J. C., Jr., Harmon, J. B., and Burgison, R. M., *ibid.*, **43**, 152(1954).
(3) Burger, A., "Medicinal Chemistry," Vol. I, Interscience Publishers, Inc., New York, 1951, p. 428.
(4) Burtner, R. R., and Cusic, J. W., *J. Am. Chem. Soc.*, **65**, 262(1943).
(5) Conant, J. B., and Wheland, G. W., *ibid.*, **54**, 1212 (1932).
(6) McEwen, W. K., *ibid.*, **58**, 1124(1936).
(7) Traube, W., and Nithack, W., *Ber.*, **39**, 227(1906).
(8) Oxley, P., and Short, W. F., *J. Chem. Soc.*, **1947**, 497.
(9) Wheatley, W. B., Fitzgibbon, W. E., Cheney, L. C., and Binkley, S. B., *J. Am. Chem. Soc.*, **72**, 4443(1950).
(10) Blicke, F. F., and Zienty, M. F., *ibid.*, **63**, 2945 (1941).
(11) Campaigne, E., and LeSuer, W. M., *ibid.*, **70**, 1555 (1948).
(12) Von, I., and Wagner, E. C., *J. Org. Chem.*, **9**, 162 (1944).
(13) Wislicenus, W., and Russ, K., *Ber.*, **43**, 2719(1910).
(14) Sieglitz, A., and Jassoy, H., *ibid.*, **54**, 2135(1921).

Cyanocobalamin (Vitamin B_{12})*

I. A Study of the Stability of Cyanocobalamin and Ascorbic Acid in Liquid Formulations

By ANDREW BARTILUCCI† and NOEL E. FOSS‡

The effect of *p*H on the stability of solutions of cyanocobalamin and ascorbic acid, alone, and in combinations, is reported. The effect of a contaminant complexing agent at one concentration on aqueous solutions of these vitamins is also reported. The stability of these vitamins, alone, and in combination, in various mixtures of propylene glycol, Sorbo, syrup, glycerin, glucose, and distilled water has been determined. The analytical methods employed for following the stability of cyanocobalamin include the U. S. P. method and the chemical method.

THERE IS A DEARTH of literature concerning the stability of vitamin B_{12} (1–5). The interest in the combination of this vitamin with ascorbic acid (6–8) justified a basic study of the incompatibility of cyanocobalamin with ascorbic acid (1). Preliminary investigations are reported indicating the effect of *p*H, the effect of a trace-contaminant complexing agent, and the effect of various vehicles on the stability of cyanocobalamin and ascorbic acid, separately and in combination.

EXPERIMENTAL

The analytical procedures used for the determination of cyanocobalamin included both the chemical method (9–11) and the U. S. P. method (12). Ascorbic acid was determined by titration in a 1% oxalic-acid solution with 0.1 *N* iodine using starch T. S. as the indicator. Generally, determinations were discontinued after about 50% decomposition had taken place.

The cyanocobalamin used in this laboratory was in the form of a 0.1% trituration with mannitol as the diluent. The ascorbic acid used in the study was of U. S. P. quality in a fine crystalline form. The water used as a solvent was distilled water redistilled from an all-glass still, unless otherwise noted. The other solvents, reagents, and salts were of official quality, unless otherwise identified. Containers, pipets, and all glassware used were thoroughly washed, rinsed several times with glass-distilled water, and air-dried before use.

The samples contained 10 ± 2 μg./cc. of cyanocobalamin and/or 50 ± 5 mg./cc. of ascorbic acid. Five-hundredths per cent of propylparaben was included in all formulations as the preservative. The filtered (glass wool) solutions were subdivided into one-ounce flint glass, screw-capped bottles and stored in cardboard boxes at refrigerated (5°), elevated (40°), and room (25–30°) temperatures.

The Effect of pH. A series of solutions was prepared using 1 *M* sodium phosphate and 0.5 *M* citric acid buffer stock solutions (13). Solutions of ascorbic acid and cyanocobalamin were prepared at several *p*H levels between 2.2 and 7.0. Maximum stability for ascorbic acid was achieved at *p*H 6.3, which is in agreement with previous reports (14–16). Under the test conditions employed, which included protection from strong light, only the cyanocobalamin sample at *p*H 2.2 showed a loss (5%) after eight months at 40°. Maximum stability of a sample containing both ascorbic acid and cyanocobalamin was exhibited at *p*H 6.3. This finding is contrary to those reported for vitamin B_{12a} and ascorbic acid (2).

The Effect of a Complexing Agent.—Tetrasodium ethylenediaminetetraacetate[1] (NaEDTA) was used

TABLE I.—COMPOSITION OF VEHICLES USED AS SOLVENTS FOR CYANOCOBALAMIN AND ASCORBIC ACID

Vehicle	Percentage by Volume					
	Propylene Glycol	Glycerin	Glass Distilled Water	Syrup U. S. P.[a]	Sorbo[b]	Glucose
A	100	...	...	...	...	...
B	75	25	...	...	...	...
C	50	50	...	...	...	...
D	75	...	25	...	...	...
E	50	...	50	...	...	...
F	25	...	...	...	75	...
G	50	...	...	...	50	...
H	75	...	...	...	25	...
I	...	...	...	...	100	...
J	...	...	...	...	75	25
K	...	25	...	...	75	...
L	...	...	25	...	75	...
M	...	...	...	25	75	...
N	...	...	...	...	50	50
O	...	50	...	...	50	...
P	...	...	50	...	50	...
Q	...	...	...	50	50	...
R	...	75	...	...	25	...
S	...	...	75	...	25	...
T	...	...	...	75	25	...

[a] Syrup U. S. P. was prepared by the cold method.
[b] Sorbo is a 70% aqueous solution of *d*-sorbitol commercially available from the Atlas Powder Company.

* Received August 18, 1952, from the Department of Pharmacy, University of Maryland School of Pharmacy, Baltimore, Md.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

† Fellow of the American Foundation for Pharmaceutical Education, 1950–52. Present address: St. John's University College of Pharmacy, Brooklyn, N. Y.

‡ The authors would like to express their appreciation to Dr. R. T. Major, Scientific Director and Vice-President, Merck & Co., Inc., for the generous supply of cyanocobalamin. We are also indebted to Drs. G. E. Boxer and W. C. Gakenheimer for helpful suggestions on the chemical method of analysis employed.

[1] Tetrasodium ethylenediaminetetraacetate was graciously supplied by the Bersworth Chemical Co. under the trade name, Versene.

TABLE II.—EFFECT OF TEMPERATURE ON STABILITY OF SOLUTIONS CONTAINING 50 ± 5 MILLIGRAMS OF ASCORBIC ACID PER CUBIC CENTIMETER

Vehicle	°C.[a]	Percentage of Ascorbic Acid Retained, Days								
		10	30	60	90	120	150	180	210	240
A	5	...	100	100	100	...	100	100	...	100
	RT	100	100	100	89.0	...	72.0	59.0	...	...
	40	100	97.7	93.9	90.6	61.8	36.2	...	...	...
B	5	...	100	100	100	...	100	100	...	100
	RT	...	100	100	100	...	99.2	91.3	88.3	...
	40	100	96.5	92.5	92.0	90.6	...	...	...	73.8
C	5	...	100	100	100	...	100	...	100	100
	RT	...	98.0	...	98.0	96.0	92.0	...	93.2	93.3
	40	100	99.8	100	88.8	86.7	...	...	...	82.0
D	5	...	100	100	100	100	...	...	100	...
	RT	...	95.8	97.9	98.0	...	91.3	90.8	89.0	...
	40	98.0	94.0	91.3	91.3	90.8	88.8	84.3	84.0	82.3
E	5	...	100	100	100	100	...	...	100	...
	RT	...	100	95.3	...	97.3	94.5	93.1	90.0	...
	40	100	93.8	91.8	86.3	72.5	59.2	...	...	19.7
F	5	...	...	Gelled	...	...	...	...	...	...
	RT	...	92.3	92.3	90.9	91.8	...	89.1	...	87.0
	40	100	100	91.5	90.3	86.3	...	...	...	80.8
G	5	Gelled	...	...	...	...	...	...	...	...
	RT	...	92.7	91.8	90.9	...	82.1	79.8	...	71.3
	40	97.3	92.5	90.5	88.0	...	76.8	72.5	71.0	62.5
H	5	...	...	100	100	...	100	Ppt.[b]	...	...
	RT	...	100	99.2	...	95.7	96.0	94.0	90.2	...
	40	100	90.8	...	88.8	86.5	83.0	83.8	79.5	...
I	5	...	...	...	...	...	100	...	100	...
	RT	...	100	100	100	100	92.7	91.8	91.5	...
	40	100	87.0	...	76.8	71.8	64.8	55.8	52.3	39.8
J	5	...	98.0	98.0	98.0	98.0	98.0	98.0	98.0	...
	RT	...	...	...	...	95.5	92.5	85.5	85.5	...
	40	97.5	87.3	81.3	78.8	76.0	68.0	64.0	61.0	57.3
K	5	...	100	100	...	...	100	...	...	...
	RT	...	90.5	87.5	90.3	88.0	87.5	86.3	85.7	...
	40	97.5	94.3	92.3	89.3	86.5	80.8	78.8	74.3	...
L	5	...	100	100	100	...	100	...	...	100
	RT	...	95.5	95.4	...	94.2	...	93.0	91.0	...
	40	100	93.5	...	82.9	76.6	67.0	62.1	54.4	50.4
M	5	...	100	100	...	...	100	...	...	100
	RT	...	100	100	...	89.2	89.2	...	76.9	...
	40	97.3	92.0	85.0	...	81.2	75.3	...	59.2	44.5
N	5	...	100	...	...	...	...	...	...	92.8
	RT	...	100	100	94.5	90.2	85.9	80.2	73.9	...
	40	100	93.0	86.7	79.0	74.0	73.0	59.5	...	...
O	5	...	100	100	...	...	100	100	...	100
	RT	...	98.2	98.4	97.8	...	95.3	...	92.1	91.4
	40	...	97.8	97.8	92.7	91.1	...	...	...	81.9
P	5	...	100	100	...	...	...	...	100	...
	RT	...	94.3	94.3	83.0	82.0	80.0	73.2	71.1	...
	40	100	87.0	73.8	61.3	49.0	37.8	...	...	...
Q	5	...	100	100	...	100	100	98.0	98.0	96.2
	RT	...	94.6	...	89.1	...	72.3	...	64.3	...
	40	89.0	87.5	81.5	78.2	73.3	68.7	58.2	52.5	...
R	5	100	...	100	...	...	...	...	...	100
	RT	100	...	98.9	98.0	97.4	97.0	...	95.1	...
	40	90.3	88.5	89.3	87.5	85.7	86.7	83.6	84.5	...
S	5	97.8	...	98.5	97.1	95.4	...	...	...	92.4
	RT	...	96.5	92.0	82.1	75.8	60.3	54.1	...	...
	40	96.3	82.8	67.0	52.6	32.5	...	...	...	...
T	5	100	100	100	100	100	...	...	...	100
	RT	...	95.1	...	90.1	84.0	80.3	70.0	51.2	...
	40	96.0	91.0	88.5	74.0	68.3	47.5	...	...	...

[a] RT = Room temperature (25–30°).

[b] Ppt.—Precipitation occurred.

at a concentration of 0.05% (17). The complexing agent was effective in stabilizing a solution of ascorbic acid prepared with water distilled from a metal still (18).

Two aqueous solutions of cyanocobalamin were prepared containing NaEDTA. One solution was made acid (*p*H *ca.* 5) with 1 *N* sulfuric acid. The unneutralized solution had a *p*H of 10.3. There was about 50% decomposition of the cyanocobalamin in the unneutralized solution after one week at 40° and one month at room temperature. The *p*H of the decomposed solution remained at 10.3. The solution made acid exhibited no change in the vitamin content after storage for two months. The vitamin content was determined spectrophotometrically using a suitable blank.

Two aqueous solutions of a mixture of cyanocobalamin and ascorbic acid were prepared. To one solution was added NaEDTA. Both solutions had a *p*H of about 2.5. After sixty days at 40°, the sample containing NaEDTA had retained 55.3% of vitamin B_{12} and 72.3% of ascorbic acid compared to 35.9% and 66.1%, respectively, in the other sample. The period of study was too short to make possible conclusions regarding the slight stabilization noted. Investigations are being continued. The cyanocobalamin content was determined by the chemical method.

The Effect of Various Vehicles.—Solutions of cyanocobalamin and ascorbic acid were prepared employing solvents most commonly used in vitamin preparations (Table I). Several of these vehicles were used to prepare solutions containing both cyanocobalamin and ascorbic acid. The cyanocobalamin determinations were made by the chemical method. A high concentration of propylene glycol with water, glycerin, or Sorbo, and a mixture of glycerin and Sorbo were found to afford maximum stability conditions for ascorbic acid (Table II). Cyanocobalamin was found to be stable in a wide variety of solvents. It has questionable stability in high concentrations of propylene glycol and in solvents containing glucose (Table III). A study of Table IV indicated that vehicle *C*, composed of equal parts, by volume, of propylene glycol and glycerin, afforded maximum stability conditions for a mixture of cyanocobalamin and ascorbic acid.

DISCUSSION

The 0.1% trituration in mannitol provided crystalline cyanocobalamin in a form which was convenient to weigh and handle. A stabilizing effect on a solution of ascorbic acid by mannitol was noted. The unsatisfactory use of liquefied phenol U. S. P. as a preservative for cyanocobalamin has been confirmed (4).

The percentage of propylparaben in the finished solutions was not known, the excess having been filtered out, but the amount present was apparently effective in inhibiting mold growth in all samples under study.

TABLE III.—EFFECT OF TEMPERATURE ON STABILITY OF SOLUTIONS CONTAINING 10 ± 2 MICROGRAMS OF CYANOCOBALAMIN PER CUBIC CENTIMETER

Vehicle	°C.[a]	Percentage of Cyanocobalamin Retained, Days 30	60
A	5	100	100
	RT	96.8	90.4
	40	88.6	65.4
D	5	100	100
	RT	100	88.2
	40	100	85.2
H	5	...	ppt.[b]
	RT	...	87.1
	40	...	82.0
J	5	83.6	...
	RT	77.2	...
	40	76.2	...
N	5	...	96.4
	RT	...	90.3
	40	...	88.9

[a] RT = Room temperature (25–30°).
[b] Ppt.—Precipitation occurred.

Although the chemical method of analysis of cyanocobalamin may have indicated no decomposition, all vehicles containing mixtures of cyanocobalamin and ascorbic acid exhibited various color changes on storage. It is believed that the color changes were a result of ascorbic acid decomposition. The observation leading to this belief was based on the ability to reproduce the color changes exhibited by solutions of cyanocobalamin and ascorbic acid, undergoing decomposition on storage, by adding ascorbic acid solutions in various stages of decomposition to an undecomposed solution of cyanocobalamin. It is known that ascorbic acid solutions, initially colorless, decompose with the production of yellow to brown solutions, depending on the extent of decomposition.

Freshly prepared solutions of cyanocobalamin and ascorbic acid are pink due to the cyanocobalamin content; the color gradually changes, however, to orange and finally to brown. The rapidity of the color change is directly related to the rate of color development in a solution of ascorbic acid with the same vehicle. A color wheel shows that the colors red (or pink from the cyanocobalamin) and yellow (decomposed ascorbic acid solution) combine to form an orange color (color of solution of cyanocobalamin and ascorbic acid after storage). Since only after the color starts turning a deeper orange toward orange-brown is there any indication of a cyanocobalamin loss by the chemical method, it is suspected that the decomposition products of ascorbic acid play an important part in the decomposition of cyanocobalamin. Table V summarizes the color changes that occur in solutions of ascorbic acid and in solutions of mixtures of cyanocobalamin and ascorbic acid on storage. The colors appearing in the bottom row of the table may be produced by combining solutions with the colors indicated in the first two rows.

SUMMARY

The optimum *p*H for stability of ascorbic acid in solution was found to be above 6.0 but below 7.0. The optimum *p*H for cyanocobalamin is between 4.5 and 5.0. The most favorable *p*H for a mixture of cyanocobalamin and ascorbic acid appears to be between 6.0 and 7.0.

Tetrasodium ethylenediaminetetraacetate has a stabilizing effect on ascorbic acid in distilled water. Unless acidified, it has a deleterious effect on cyanocobalamin. A slight stabilization was noted at elevated temperature on the active ingredients when the complexing agent was included in a mixture of cyanocobalamin and ascorbic acid.

Ascorbic acid was found to be most stable in high concentrations of propylene glycol with distilled water, glycerin or Sorbo, and a mixture of glycerin and Sorbo. Cyanocobalamin was found to be stable in a wide variety of vehicles. It has questionable stability in high concentrations of propylene glycol and in vehicles contain-

TABLE IV.—EFFECT OF TEMPERATURE ON STABILITY OF SOLUTIONS CONTAINING 10 ± 2 MICROGRAMS OF CYANOCOBALAMIN AND 50 ± 5 MILLIGRAMS OF ASCORBIC ACID PER CUBIC CENTIMETER

Vehicle	°C[a]	Percentage Retained, Days											
		30		60		90		120		150		180	
		B_{12}[b]	AA[c]	B_{12}	AA	B_{12}	AA	B_{12}	AA	B_{12}	AA	B_{12}	AA
A	5	100	100	100	100	100	100	100	99.3	100	96.2	100	93.5
	RT	...	96.5	99.6	93.8	90.3	87.5	...	83.5	77.3	81.0	...	72.5
	40	100	95.8	90.3	95.9	85.0	89.8	76.8	79.5	63.7	55.9	...	28.0
B	5	100	100	100	100	100	100	100	100	100	99.2	100	96.8
	RT	100	100	100	100	100	97.0	100	89.1	100	84.4	94.0	89.2
	40	100	93.5	91.0	91.0	...	90.4	...	77.5	90.7	76.7	75.3	69.2
C	5	100	100	100	100	100	100	100	98.2	100	98.2	100	98.1
	RT	100	100	100	100	100	95.2	100	92.4	100	93.7	100	92.4
	40	100	100	100	100	100	87.0	100	91.3	100	89.7	81.0	84.5
D	5	100	98.1	100	100	100	99.5	100	90.3	...	...	100	90.6
	RT	100	98.5	100	96.8	100	95.4	100	89.5	...	...	93.6	84.5
	40	100	92.4	100	58.4	100	88.9	87.7	85.4	...	...	77.6	81.7
E	5	100	100	100	...	100	100	...	98.9	...	94.1	95.3	95.1
	RT	100	100	96.1	100	85.8	...	83.8	94.8	82.6	89.6	82.6	84.8
	40	100	99.0	88.5	95.5	85.8	...	82.0	86.3	82.3	78.9	76.5	73.3
F	5	...	Gelled	...	...	...	...	...	...	...	...	...	...
	RT	100	98.5	100	89.0	100	87.0	93.1	83.6	81.3	77.6	...	...
	40	100	88.5	100	74.0	90.2	64.8	85.3	62.5	65.2	54.2	...	...
G	5	...	100	...	Gelled	...	...	...	...	...	...	...	...
	RT	100	100	100	91.2	100	88.6	96.5	76.9	82.7	71.9	...	...
	40	99.6	99.7	94.3	81.4	89.6	75.9	78.2	64.3	74.8	54.8	...	...
I	5	100	100	96.8	100	92.7	100	92.6	92.5	92.7	...	88.9	89.7
	RT	86.7	91.0	72.3	89.5	57.9	87.0	48.3	80.5	...	...	36.5	69.7
	40	58.2	82.2	38.7	77.4	...	75.7	...	59.4	...	...	...	...
J	5	55.2	90.7	Ppt.[d]	...	...	...	...	...	...	...	...	...
	RT	29.5	85.5	...	...	...	84.2	...	...	...	...	...	...
	40	0.0	82.9	...	...	...	70.5	...	...	...	...	...	...
M	5	...	100	94.9	100	...	...	...	...	...	...	...	...
	RT	71.2	98.5	54.7	96.8	...	...	...	...	...	...	...	...
	40	26.6	77.0	...	73.5	...	...	...	...	...	...	...	...

[a] RT = Room temperature (25–30°). [c] Ascorbic acid.
[b] Cyanocobalamin. [d] Precipitation occurred.

TABLE V.—SUMMARY OF COLOR CHANGES IN SAMPLES OF ASCORBIC ACID AND CYANOCOBALAMIN–ASCORBIC ACID SOLUTIONS IN PROGRESSIVE STAGES OF DECOMPOSITION

	Initial	1	2	3	4	5
Cyanocobalamin	Pink	Pink	Pink	Pink	Pink	Pink
Ascorbic acid	Colorless	Slight yellow	Yellow	Orange	Brownish orange	Brown
Cyanocobalamin–ascorbic acid	Pink	Peach	Yellowish orange	Orange	Brownish orange	Brown

ing glucose. A vehicle composed of equal parts of propylene glycol and glycerin afforded maximum stability conditions for a mixture of cyanocobalamin and ascorbic acid. There was about 80% retention of cyanocobalamin and 85% retention of ascorbic acid in this vehicle after six months' storage at 40°. There was no loss of cyanocobalamin and about 10% loss of ascorbic acid on storage for six months at room temperature.

It is suggested that the decomposition products of ascorbic acid play an important part in the decomposition of cyanocobalamin. Investigations are continuing on various phases of this problem, and it is expected that the results will be published.

REFERENCES

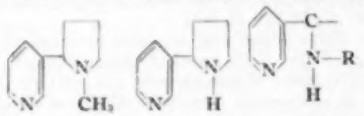
(1) Gakenheimer, W. C., and Feller, K. A., THIS JOURNAL, **38**, 660(1949).
(2) Trenner, N. R., Buhs, R. P., Bacher, F. A., and Gakenheimer, W. C., *ibid.*, **39**, 361(1950).
(3) Hartley, F., Stross, P., and Stuckey, R. E., *J. Pharm. Pharmacol.*, **2**, 648(1950).
(4) Macek, T. J., and Feller, K. A., THIS JOURNAL, **41**, 285(1952).
(5) "Crystalline Vitamin B_{12} Merck U. S. P.," *Merck Service Bulletin*, Merck & Co., Inc., Rahway, N. J., 1950.
(6) Dyke, S. C., Della Vida, B. L., and Kelidet, E., *Lancet*, **2**, 278(1942).
(7) May, C. D., *et al.*, *Bull. Univ. Minn. Hosp.*, **21**, 208 (1950).
(8) Glass, G. B. J., *et al.*, *Science*, **115**, 101(1952).
(9) Boxer, G. E., and Rickards, J. C., *Arch. Biochem.*, **30**, 372(1950).
(10) *Ibid.*, **30**, 382(1951).
(11) *Ibid.*, **30**, 392(1951).
(12) U. S. P., XIV, p. 661.
(13) Lange, N. A., "Handbook of Chemistry," Handbook Publishers, Inc., Sandusky, Ohio, 1946.
(14) Ganguly, S. K., *Indian J. Pharm.*, **11**, 145(1949); through *Chem. Abstr*, **44**, 1647(1950).
(15) Ciminera, J. L., and Wilcox, P. W., THIS JOURNAL, **35**, 363(1946).
(16) "Ascorbic Acid," *Merck Vitamin Reviews*, Merck & Co., Inc., Rahway, N. J., 1949, p. 12.
(17) "The Versenes," *Technical Bulletin No. 2*, Bersworth Chemical Co., Framingham, Mass., 1951.
(18) Jäger, H., *Pharmazie*, **3**, 536(1948).

The Synthesis of Possible Degradation Products of Nicotine*

By RAYMOND N. CASTLE† and ALFRED BURGER

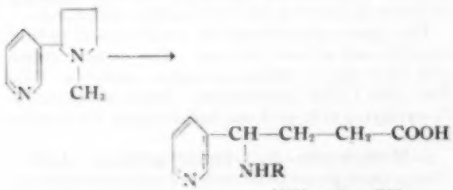
The following compounds have been synthesized as possible degradation products of nicotine: γ-keto-γ-(3-pyridyl)butyric acid, β-amino-β-(3-pyridyl)-propionic acid, and β-methylamino-β-(3-pyridyl)-propionic acid. Certain derivatives of these compounds have been prepared.

LARSON, HAAG, and others (1–4) have been interested in the biological fate of nicotine (I). It has been found (4) that the urine of dogs that had received nicotine contained a substance which gave a red color with cyanogen bromide, while the color reaction was not observed if the dogs had not received nicotine. The urine of dogs that had received nornicotine (II) also gave a red color with cyanogen bromide. This color was due to unchanged nornicotine. It has been established (5) that nornicotine is not a metabolite of nicotine. In order to determine the structural requirements for the red color with cyanogen bromide, a series of compounds was examined and it was found that a red color was obtained when a primary or secondary amino group was attached to the carbon atom adjacent to the pyridine ring (III), (where R may be H or alkyl). This would suggest enzymic cleav-



(I) (II) (III)

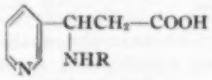
age of the pyrrolidine ring of nicotine between carbon atom five and the nitrogen atom as shown in Fig. 1. This is in accord with the mechanism postulated by Taggart and Krakaur (6) for the enzymic cleavage of the pyrrolidine ring of proline and hydroxyproline.



(IV) a, R = CH_3
(IV) b, R = H

Figure 1

The corresponding propionic acid analogs of IV are also possible degradation products of nicotine. We have synthesized β-amino-β-(3-pyridyl) propionic acid V_a by the addition of hydroxylamine to β-(3-pyridyl)acrylic acid. V_a was also prepared by the condensation of nicotinaldehyde, malonic acid, and ammonium acetate in absolute ethanol. It was accompanied by β-(3-pyridyl)



(V) a, R = H
(V) b, R = CH_3

acrylic acid and by a large amount of an unidentified substance which could be converted to the above acid by heat. β-Methylamino-β-(3-pyridyl)propionic acid V_b was prepared by the condensation of nicotinaldehyde, malonic acid, and methylammonium acetate in absolute ethanol. Both V_a and V_b gave a red color with cyanogen bromide (5). Compounds V_a and V_b have been characterized by their infrared absorption spectra[1] obtained in a mineral oil mull. Both amino acids show absorption bands in the 6.0–6.7 μ region which are characteristic of amino acids.

For the attempted synthesis of γ-amino-γ-(3-pyridyl)butyric acid IV_b, ethyl nicotinate VI was condensed with diethyl succinate VII by a modification of the procedure of Shivers, Dillon, and Hauser (7). The diethyl α-nicotinoylsuccinate VIII thus obtained was converted to γ-keto-γ-(3-pyridyl)butyric acid IX by acid hydrolysis and decarboxylation (Fig. 2).

After the synthesis of IX was completed Wada and Yamasaki (8) announced the isolation of the keto acid IX from microbial culture media in which nicotine was the only nitrogen-containing substrate. Our synthesis constitutes a proof of structure of their keto acid. It is entirely possible that this compound may be important in other biological degradations of nicotine, although it would not be expected to give a red color with cyanogen bromide.

* Received August 21, 1953, from The Cobb Chemical Laboratory, University of Virginia, Charlottesville, Va.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
This work was supported by a grant from the American Tobacco Company, Research Laboratory, Richmond, Va.
† Box 93, The University of New Mexico, Albuquerque, N. M.

[1] These spectra were determined by E. S. Harlow and W. B. Wartman of the Research Laboratory of the American Tobacco Company, Richmond, Va.

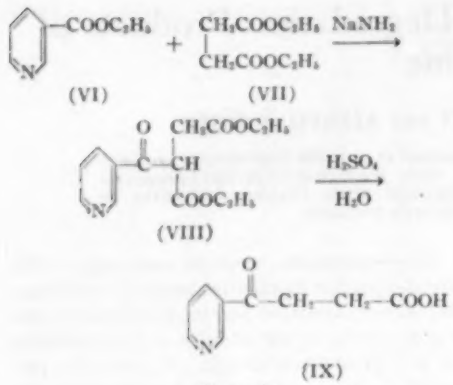
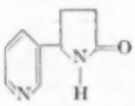


Figure 2

Compound IX was readily converted to the oxime. All attempts to reduce the oxime to the amino acid IV_b or to the lactam X have been unsuccessful. We were also unable to convert the keto acid IX into IV_a or X by catalytic reduction in the presence of ammonia. It was expected



(X)

that the reduction of the acetylhydrazone of IX would furnish IV_a or X since the acetylhydrazone of diethyl β-ketopimelate was a good source of β-aminopimelic acid (9). However, the reduction of the acetylhydrazone of IX with aluminum amalgam gave unidentifiable gums. Other synthetic routes to IV_a, IV_b, and X are being investigated.

In Table I the important features of the ultraviolet absorption spectra of IX and its methyl ester are compared with those of 3-acetylpyridine (10) and with the natural keto acid isolated by Wada and Yamasaki (11).

Table I.—Comparison of Ultraviolet Absorption Spectra

Compound	Maxima λ, mμ	Maxima ε × 10⁻³	Minima λ, mμ	Minima ε × 10⁻³
3-Acetylpyridine	228	8.48	250	2.05
	267	2.88		
γ-Keto-γ-(3-pyridyl)-butyric acid	227.5	9.18	250	2.08
	267.5	3.09		
Methyl γ-keto-γ-(3-pyridyl)butyrate	229	9.42	250	2.30
	267.5	3.63	300	0.23
	310	0.38	320	0.13
Natural Keto Acid (11)	228.5	8.63	250	...
	267	2.97	...	...

EXPERIMENTAL

All melting points are uncorrected.

β-(3-Pyridyl)acrylic Acid.—This compound was prepared by the method of Panizzon (12) from nicotinaldehyde[1] and malonic acid in the presence of pyridine and piperidine, m. p. 231–234°.

β-(3-Pyridyl)acrylic Acid Hydrobromide.—It was prepared by refluxing the β-(3-pyridyl)acrylic acid in 40% aqueous hydrobromic acid. The nearly pure hydrobromide separated as white plates upon cooling. A sample purified for analysis melted at 263–265°.

Anal.—Calcd. for $C_8H_7NO_2 \cdot HBr$: C, 41.76; H, 3.51. Found: C, 42.17; H, 3.75.

[1] Supplied by Dr. John Aeschlimann, Hoffmann-La Roche, Nutley, N. J.

β-(Amino-β-(3-pyridyl)propionic Acid.—The procedure in *Organic Syntheses* (13) for the preparation of β-phenyl-β-alanine was adapted to the preparation of the 3-pyridyl analog.

To a hot solution of sodium ethoxide, prepared from 2.3 Gm. (0.1 Gm. atom) of sodium and 80 ml. of absolute ethanol, was added with shaking 6.95 Gm. (0.1 mole) of hydroxylamine hydrochloride. The suspended sodium chloride was filtered off and washed with absolute ethanol. To this solution was added 7.45 Gm. (0.05 mole) of β-(3-pyridyl)-acrylic acid. A voluminous precipitate separated which dissolved upon heating. After nine hours of refluxing the reaction mixture deposited 0.6 Gm. of the desired amino acid upon cooling. After repeated purification, accomplished by dissolving the compound in water and precipitating it with absolute ethanol, it gave fine white plates, m. p. 206° (dec.).

Anal.—Calcd. for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07. Found: C, 58.18; H, 6.11.

The above amino acid was also prepared by refluxing 3.2 Gm. (0.03 mole) of nicotinaldehyde, 3.1 Gm. (0.03 mole) of malonic acid, and 4.6 Gm. (0.06 mole) of ammonium acetate in 25 ml. of absolute ethanol. The evolution of carbon dioxide which began upon mixing the reactants continued for nearly the entire refluxing period of one hour. After allowing the reaction mixture to cool, the white solid (2.8 Gm.) was filtered and dried. This substance decomposes with the evolution of gas (both carbon dioxide and ammonia) at 148–160° and is converted into β-(3-pyridyl)acrylic acid, m. p. 231–234°. After several purifications this extremely water-soluble substance would not give a satisfactory analysis for any possible compound.

The filtrate was refluxed for an additional twenty minutes and allowed to cool. The desired amino acid (0.6 Gm.), which separated, melted at 205–206° (dec.) after purification. Some additional β-(3-pyridyl)acrylic acid was isolated from the mother liquor.

β-Methylamino-β-(3-pyridyl)propionic Acid.—Thirty-three grams (0.36 mole) of methylammonium acetate, 18.6 Gm. (0.18 mole) of malonic acid, and 19.2 Gm. (0.18 mole) of nicotinaldehyde were dissolved in 150 ml. of absolute ethanol and the mixture refluxed for two hours. On cooling the solution, 9.2 Gm. of β-(3-pyridyl)acrylic acid separated. The filtrate solidified to a mush which altogether produced 6.2 Gm. of β-methylamino-β-(3-pyridyl)-propionic acid. A sample purified for analysis by several crystallizations from absolute methanol melted at 171–171.5°.

Anal. Calcd. for $C_9H_{12}N_2O_3$: C, 59.98; H, 6.72. Found: C, 59.43; H, 6.78.

This procedure has been carried out several times. The yield of the amino acid varies with no apparent changes in procedure. In two experiments only β-(3-pyridyl)acrylic acid could be isolated.

γ-Keto-γ-(3-pyridyl)butyric Acid.—To 16.4 Gm. (0.42 mole) of sodium amide, covered with dry ether, were added all at once 69.7 Gm. (0.4 mole) of freshly distilled diethyl succinate and then 33.3 Gm. (0.22 mole) of ethyl nicotinate. Sufficient dry ether was added to bring the total volume of ether to 80 ml. If more ether was used the yields were negligible. The mixture was refluxed and stirred for two and one-half hours when the evolution of ammonia was completed.

The thick brown reaction mixture was poured into a mixture of ice and concentrated hydrochloric acid. The acid mixture was then saturated with solid sodium bicarbonate and repeatedly extracted with ether. This ether extract (*A*) was extracted several times with 5% hydrochloric acid; the acid extract was again saturated with sodium bicarbonate and extracted with ether. After drying the extract (anhydrous sodium sulfate), the ether was evaporated. From the residual oil 8.6 Gm. of ethyl nicotinate was recovered. On fractionation 22.6 Gm. (50%) of diethyl α-nicotinoyl-succinate boiling at 160–162° at 1 mm. was obtained. The maximum yield obtained in other experiments amounted to 56%. From ether extract *A* some diethyl succinate was always recovered together with some diethyl 2,5 diketo-1,4-cyclohexanedicarboxylate, m. p. 124°.

The diethyl α-nicotinoylsuccinate was hydrolyzed and decarboxylated by refluxing a mixture of 15 Gm. of the ester with 26 ml. of water and 2 ml. of concentrated sulfuric acid for forty-eight hours. After cooling and adjustment of the solution to *p*H 4–4.5 with ammonium hydroxide, the γ-keto-γ-(3-pyridyl)butyric acid separated. There was obtained 7.3 Gm. (76%) of compound which after four crystallizations from ethanol melted at 161.5–163°.

Anal. Calcd. for $C_9H_9NO_3$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.34; H, 5.20; N, 7.63.

γ-Oximino-γ-(3-pyridyl)butyric Acid.—To 9.0 Gm. (0.05 mole) of γ-keto-γ-(3-pyridyl)butyric acid in 50 ml. of 10% sodium hydroxide solution was added 5.25 Gm. (0.075 mole) of hydroxylamine hydrochloride dissolved in 15 ml. of water. The solution, after standing forty-eight hours at room temperature, deposited 8.5 Gm. (88%) of the oxime upon neutralization with dilute sulfuric acid. The white needles melted at 165–166° after three crystallizations from hot water.

Anal. Calcd. for $C_9H_{10}N_2O_3$: C, 55.66; H, 5.19. Found: C, 55.45; H, 5.22.

Acetylhydrazone of γ-Keto-γ-(3-pyridyl)butyric Acid.—A solution of 3.58 Gm. (0.02 mole) of γ-keto-γ-(3-pyridyl)butyric acid and 1.24 Gm. (0.02 mole) of acetylhydrazine in 60 ml. of absolute ethanol was refluxed for one and one-fourth hours. About two-thirds of the ethanol was removed by distillation. The white crystalline solid (3.7 Gm.) separated on cooling. After three crystallizations from absolute ethanol the compound melted at 195–198°.

Anal. Calcd. for $C_{11}H_{13}N_3O_3$: C, 56.16; H, 5.57. Found: C, 55.97; H, 5.51.

Methyl γ-Keto-γ-(3-pyridyl)butyrate.—Five Gm. (0.028 mole) of γ-keto-γ-(3-pyridyl)butyric acid was refluxed in 100 ml. of absolute methanol containing 10 ml. of concentrated sulfuric acid for seven hours. One-half of the methanol was removed by distillation, the residue neutralized with ammonium hydroxide, and the ammonium sulfate removed by filtration. The mother liquor was concentrated to a thick syrup which solidified to a purple solid on refrigeration, amounting to 2.45 Gm. (46%). After two crystallizations (Norite) from hot water, the ester melted at 65.5–67.5°.

Anal. Calcd. for $C_{10}H_{11}NO_3$: C, 62.16; H, 5.74. Found: C, 61.91; H, 5.91.

Ultraviolet Absorption Spectra.—These data (Table I) were determined in 95% ethanol with the Beckman D. U. spectrophotometer.

REFERENCES

(1) Haag, H. B., and Larson, P. S., *J. Pharmacol. Exptl. Therap.*, **76,** 235(1942).
(2) Larson, P. S., and Haag, H. B., *ibid.*, **76,** 240(1942).
(3) Larson, P. S., and Haag, H. B., *ibid.*, **77,** 343(1943).
(4) Larson, P. S., Haag, H. B., and Finnegan, J. K., *ibid.*, **86,** 239(1946).
(5) Larson, P. S., private communication.
(6) Taggart, J. V., and Krakaur, R. B., *J. Biol. Chem.*, **177,** 641(1949).
(7) Shivers, J. C., Dillon, M. L., and Hauser, C. R., *J. Am. Chem. Soc.*, **69,** 119(1947).
(8) Wada, E., and Yamasaki, K., *Science*, **117,** 152(1953).
(9) Birkofer, L., and Storch, I., *Chem. Ber.*, **86,** 32 (1953).
(10) Swain, M. L., Risner, A., Woodward, C. F., and Brice, B. A., *J. Am. Chem. Soc.*, **71,** 1341(1949).
(11) Wada, E., and Yamasaki, K., *J. Am. Chem. Soc.*, **76,** 155(1954).
(12) Panizzon, L., *Helv. Chim. Acta*, **24,** 24E(1941).
(13) Smith, L. I., "Organic Syntheses," Vol. 22, John Wiley & Sons, New York, 1942, p. 26.

Cardiovascular Actions of Some Simple Nucleic Acid Derivatives*

By VIRGINIA SYDOW and RAYMOND P. AHLQUIST

Actions of a large number of nucleic acid derivatives were studied on the aortic pressure pulse, arterial pressure, femoral artery flow, and the electrocardiogram of anesthetized dogs, and on the rate and amplitude of contraction and coronary outflow of perfused rabbit hearts. Of the compounds examined only adenosine-3-phosphoric acid, adenosine-5-phosphoric acid, and guanylic acid had significant cardiovascular activity in the doses used. Some general relationships between chemical structure and vascular activity are presented.

WORK ON THE LIBERATION of adenyl compounds in response to traumatic shock and cobra venom, their presence in fresh defibrinated blood, and their toxic actions (1–9) interested us in re-examining their cardiovascular effects.

An excellent review of the physiological activity of nucleic acids and their derivatives was compiled by Drury in 1939 (10). Since his material still represents most of our knowledge about the physiological activity of these compounds only a brief background is given here.

Among the effects of adenosine and muscle adenylic acid (in dogs) are bradycardia, increased coronary flow, and lowered arterial pressure "due in part to cardiac slowing and in part to general arterial dilation" (11). The consensus of later opinion is that dilation is the major cause of the lowered pressure. Wedd and Drury (12) observed that, after intravenous injection in dogs, adenosine and muscle adenylic acid had similar effects on the coronary flow and mean blood pressure, both in degree and duration, while yeast adenylic acid and cytidylic acid produced a greater flow and had a longer duration of action. They also concluded that, since increased coronary flow sometimes occurred when there was no change in general blood pressure, the coronary vessels were more sensitive to these substances than blood vessels in other parts of the body. Flössner (13) stated that physiological activity did not depend on the presence of side chains on the purine ring as simple pentosides and purine bases were active. He found adenosine to have the most effect on the blood pressure. Other investigators (14–17) found purine bases and also certain nucleosides and nucleotides inactive with respect to blood pressure and flow; various organs and species were used. The greatest discrepancies occur in the guanine series which has been found active by some investigators and inactive by others and in the degree of activity of yeast adenylic acid and cytidylic acid. Most results indicate that yeast adenylic acid has little influence on the heart rate but is a strong depressor substance (10).

Experiments on the perfused rabbit heart showed the same general type of results: increased coronary flow, decreased heart rate, heart block, and decreased amplitude of contraction (14) with the adenyl compounds, adenosine, muscle, and yeast adenylic acids; contradictory results are reported for other nucleic acid derivatives.

It is of interest that the human heart is, apparently, unique and responds to adenosine, muscle, and yeast adenylic acids with an increase followed by a short-lasting decrease in rate according to Hartman (18).

In some of the work mentioned above there has been a possible doubt as to the purity of the compounds used and, in a few cases, of the actual identity. It was therefore decided to study the actions of a group of nucleic acid derivatives on the aortic pressure pulse, arterial pressure, femoral arterial flow, heart rate, and electrocardiogram of the anesthetized dog and on the coronary flow, rate, and amplitude of contraction of the perfused rabbit heart and attempt to draw some conclusions as to the order of activity and the relationship of structure to activity among them.

EXPERIMENTAL

Materials Studied.—The drugs used and an indication of their structural interrelationships are shown in Table I.

Cardiovascular Actions in Dogs.—Dogs of either sex were used. All were premedicated with morphine sulfate, 10 mg./Kg. given subcutaneously, and anesthetized with intravenous pentobarbital sodium, 20 mg./Kg. When atropine sulfate was used the dosage was 0.5 mg./Kg. Doses of the experimental drugs are expressed as free acid or base on a per kilogram basis.

Blood Flow in Anesthetized, Atropinized Dogs.—Femoral arterial blood flow was measured with an optically recording rotameter. Intra-arterial, intra-aortic, or intravenous injections of the drugs were tested, but the intra-arterial route was considered the most significant for flow studies. All drugs listed

* Received September 26, 1953, from the Department of Pharmacology, Medical College of Georgia, Augusta, Ga.
This work was supported in part by a Therapeutic Research Grant by the American Medical Association.

TABLE I.—DRUGS USED AND THEIR STRUCTURAL INTERRELATIONSHIPS

D-Ribose	D-Pentose
Adenine	6-Aminopurine
Adenosine	Adenine-9-riboside
3-Adenylic acid[a]	Adenosine-3-phosphoric acid
5-Adenylic acid[a]	Adenosine-5-phosphoric acid
Guanine	2-Amino-6-hydroxypurine
Guanosine	Guanine-9-riboside
Guanylic acid[a]	Guanosine-3-phosphoric acid
Hypoxanthine	6-Hydroxypurine
Inosine	Hypoxanthine-9-riboside
Xanthine	2,6-Dihydroxypurine
Cytosine	2-Hydroxy-6-aminopyrimidine
Cytidine	Cytosine-3-riboside
Cytidylic acid[a]	Cytidine-3-phosphoric acid
Uracil	2,6-Dihydroxypyrimidine
Uridine	Uracil-3-riboside
Thymine	5-Methyluracil

[a] Acid neutralized with sodium bicarbonate before use.

in Table I were tested for peripheral vascular effects and those showing appreciable activity were studied more closely.

Adenosine, 0.022 mg. (0.8×10^{-4} mM), 3-adenylic acid and 5-adenylic acid, 0.2 mg. (5.5×10^{-4} mM), administered intra-arterially, approximately doubled the femoral blood flow without affecting the pressure. The flow returned to the control level in one-half to two minutes. Guanylic acid, in equimolar dose, produced an increase in flow about one-sixth that of the 3-adenylic acid. Guanosine, solubilizing with difficulty, was used in both sodium hydroxide and hydrochloric acid solutions. Both increased and decreased flows were obtained depending apparently on the condition of the dog, and no conclusion as to the activity of guanosine could be made. Minimal doses of guanosine which produced an effect varied from 0.8 mg. to 6.2 mg. (2.8×10^{-3} mM to 2.2×10^{-2} mM). Equal volumes of the solvents were without pronounced effects. None of the other compounds showed activity. Figure 1 illustrates the type of flow responses obtained.

Blood Pressure in Anesthetized Dogs.—Arterial pressure was recorded from either the femoral or carotid artery by means of a mercury manometer or a Statham pressure transducer (19). Single doses or continuous infusions were administered intravenously, intra-arterially, or intra-aortically. Injections into the arch of the aorta produced peripheral vascular effects before direct cardiac effects became apparent. This served as a convenient way to separate roughly the peripheral and cardiac effects of the drugs.

A fall in arterial pressure was produced by adenosine, 3-adenylic acid, 5-adenylic acid, and guanylic acid on the injection by any route of a single dose of 2 to 10 times the amounts necessary to produce increased flow on intra-arterial injection. Equimolar doses of adenosine and 3-adenylic acid produced the same fall in arterial pressure. 5-Adenylic acid was about one-third more active and guanylic acid about one-sixth as active as adenosine. The pressure returned to the control level in one-half to one and one-half minutes after adenosine, one to three minutes after 5-acid and in five to ten minutes after 3-acid. Adenosine, 0.35 mg. (1.3×10^{-3} mM), produced the same decrease in arterial pressure after both intra-aortic and intravenous injection.

Constant injection of adenosine (0.5 cc./min. of a 4.5 mg./cc. solution) decreased the pressure for approximately one minute but with this dosage the pressure returned to the control level even though

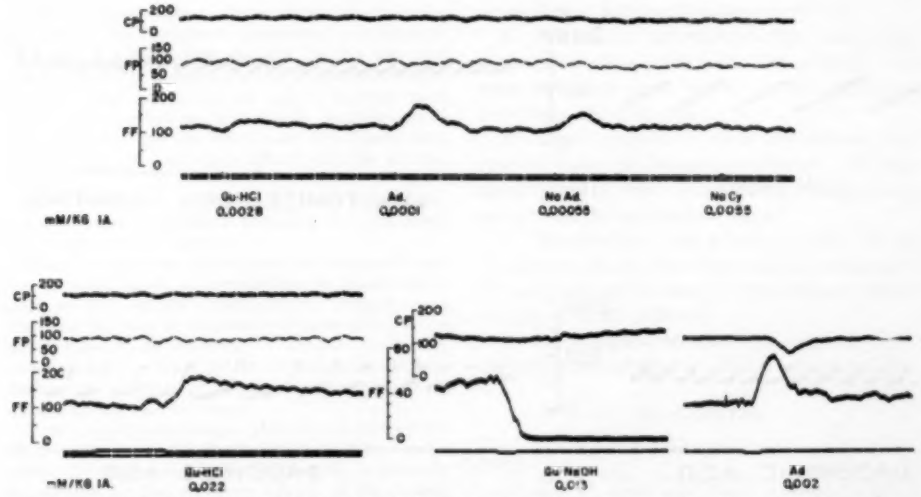


Fig. 1.—Effect of intra-arterial injection of adenyl compounds on femoral flow (cc./min.), femoral and carotid pressures (mm. of Hg.).

Gu HCl—guanosine in HCl solution; *Ad*—adenosine; *NaAd*—sodium-3-adenylate; *NaCy*—sodium cytidylate; *Gu NaOH*—guanosine in NaOH solution; *Ad 0.002*—adenosine in larger dose showing typical depressor response.

the injection was continued. Larger doses of adenosine could not be tried due to the insolubility of adenosine and the limited capacity of the injector used. Comparison of constant injection of 5-adenylic acid (40 mg./cc.) and 3-adenylic acid (40 mg./cc.) at rates which were automatically adjusted (20) to maintain a pressure equilibrium below the control pressure of the animal gave the following results: a longer time and a greater quantity of the drug required for 3-adenylic acid to reach equilibrium than for 5-adenylic acid. However, after equilibrium was reached, a slower rate of injection of the 3-acid than of the 5-acid was required to maintain the low pressure. After cessation of the injections, the pressure required two to four minutes to return to the control level after the 3-acid and fifteen to twenty seconds after the 5-acid.

The depressor effect of the active compounds was not blocked by vagotomy, piperoxan, 4 mg., tripelennamine, 10 mg., or atropine, 0.5 mg.

Electrocardiogram, Aortic Pressure Pulse, and Heart Rate in Dogs.—Observations were made on anesthetized dogs by means of either a two-channel electrocardiograph or a cathode ray cardiotachometer. The aortic pressure pulse was recorded from the arch of the aorta by means of a Hamilton or Statham manometer. Administration of drugs was by the previously described modes.

Single doses of 2–4 mg. (5.7×10^{-3} to 1.1×10^{-2} mM) of adenosine and the two adenylic acids gave observable effects on the heart rate. The effect on the electrocardiogram was small and consisted of a slight shift in the frontal plane T vector coincident with the pressure drop.

The effect on the heart rate depended on the condition and pretreatment of the animal. In unatropinized dogs with vagi intact a tachycardia of two to four times the normal control rate occurred during the pressure drop and persisted until the pressure returned to normal. This was true for both single doses and constant injection. Following atropine or vagal section adenosine and 5-adenylic acid slowed the heart rate to the pre-atropine control level or below for the duration of the depressor effect. Figure 2 illustrates these effects. The 3-adenylic acid slowed the heart rate to approximately one and one-half times the control rate for the duration of the depressor effect and thus for a longer time than the above two drugs. Smaller single doses than those stated above had no effect on the heart rate. In the constant injection experiments done, the heart was not slowed. This was probably due to the fact that the drugs were being given in smaller quantities than are necessary for direct cardiac action.

Rabbit Hearts Perfused by the Langendorff Method.—Rabbit hearts were perfused in a glass apparatus (21) and the coronary flow, heart rate, and amplitude of contraction were recorded. Drugs were injected into the coronary inflow and not allowed to recirculate or were placed in the 400-cc. reservoir and allowed to remain in the perfusion fluid. Doses are expressed as total amount of free acid or base used. Adenosine, 3-adenylic acid, and 5-adenylic acid exhibited activity.

Adenosine in doses ranging upward from 0.5 mg. injected into the coronary inflow stopped the heart for one to five seconds. The heart then resumed beating at a slow rate and with decreased amplitude. In thirty seconds to five minutes, depending on dose size, the rate returned to the control value and control or slightly greater amplitude. An irregular or weak beat was usually changed to a strong regular beat by the drug. 3-Adenylic acid and 5-adenylic acid even in doses of 5–10 mg. only slowed the heart without stopping it but otherwise acted like adenosine. The 3-acid had the least effect on the heart.

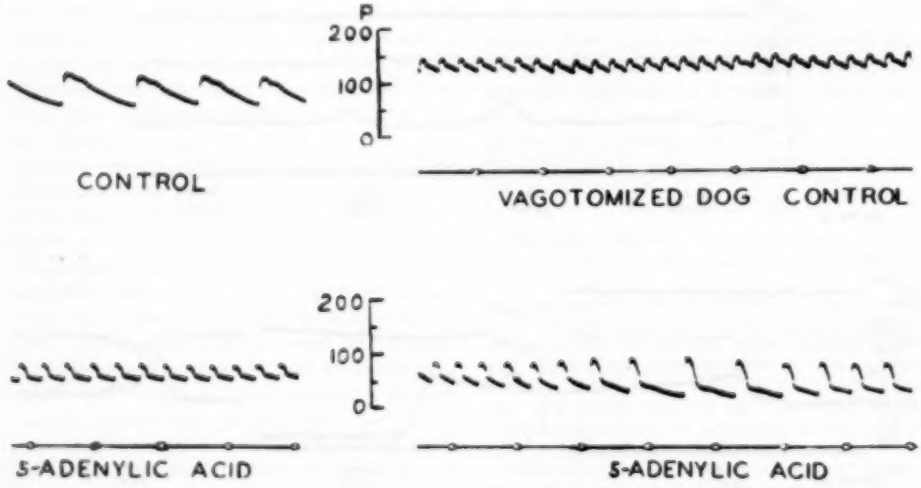


Fig. 2.—Effect of 5-adenylic acid on aortic pressure pulse (mm. Hg.) and heart rate of the dog.
Upper left—control sinus rhythm; *lower left*—depressor effect and tachycardia produced by sodium-5-adenylate (4 mg./kg.); *upper right*—tachycardia following bilateral vagal section; *lower right*—depressor effect and bradycardia following sodium-5-adenylate (4 mg./kg.). (Note the increased pulse pressure indicating no impairment of myocardial activity.)

TABLE II.—COMPARISON OF VASODILATOR ACTIVITY IN THE ANESTHETIZED DOG

Compounds	Structural Relationship	Vasodilator Activity		
		Blood Flow	Depressor Degree	Depressor Duration
Adenine	6-Aminopurine	0	0	0
Adenosine	Adenine-9-riboside	++++	++	+
3-Adenylic acid	Adenosine-3-phosphoric acid	++	++	++++
5-Adenylic acid	Adenosine-5-phosphoric acid	++	+++	++
Guanine	2-Amino-6-hydroxypurine	0	0	0
Guanosine	Guanine-9-riboside	?	...	...
Guanylic acid	Guanosine-3-phosphoric acid	+*	+*	...
Cytidylic acid	Cytidine-3-phosphoric acid	(+)	...	...
All others	.....	0	0	0

* One-sixth of 3-adenylic acid.

The effect on coronary flow was erratic and could not be correlated with the size of the dose of material. There was at no time a greater than 15% increase or decrease in flow.

Doses of less than 4.5 mg. of any of the drugs introduced into the 400-cc. reservoir for prolonged perfusion had no effect on the rate, amplitude, or coronary flow. Doses of 4.5 mg. or more markedly slowed the heart rate for as long as the perfusion continued. The amplitude of contraction was first slightly decreased and then gradually returned to normal or greater than normal. No attempt was made to continue such a perfusion for longer than three hours but at least for that period of time the hearts remained small with a strong regular beat. The effect on coronary flow was again erratic and small.

The myocardial and coronary flow effects were not significantly modified by simultaneous or previous injection of oxytocin, vasopressin, epinephrine, or atropine.

DISCUSSION

The depressor effect of the active adenyl compounds is due to direct peripheral vasodilation. This fact is clearly demonstrated by the increased femoral blood flow following intra-arterial administration. The similarity of the response with either intravenous or intra-aortic administration also indicates that the heart does not significantly contribute to the depressor effect with the smaller doses.

The tachycardia produced by these compounds in unatropinized animals is probably of reflex origin due to the fall in pressure.

Sinus bradycardia is produced by adenosine and the adenylic acids in atropinized or vagotomized dogs, or the perfused rabbit heart. Except for a transient decrease in amplitude of contraction of the isolated heart no evidence of cardiac weakening was found. An examination of the pulse contour during the bradycardia gives no indication of any significant myocardial effect.

Drury and Szent-Gyorgyi (11) have described the bradycardia produced by the 3- and the 5-adenylic acids. In their opinion the bradycardia is partially responsible for the depressor effects of these compounds. In our studies, the fall in arterial pressure was apparently independent of the heart rate.

The erratic effect on coronary flow was unexpected in view of the work of Sherrod and Louis (22) and numerous other investigators and may have been due to a fault inherent in the preparation.

The limited list of active compounds, adenosine, 3-adenylic acid, 5-adenylic acid, and guanylic acid gives only a slight indication of the structural requirements for vasodilator activity in this class of compounds. As shown in Table II, it appears that the ribose residue is necessary but not sufficient for activity, an amino group on the purine or pyrimidine ring is necessary, and the position modifies the activity.

Where a phosphoric acid residue is present the position affects activity. The delay in reaching maximum effect with 3-adenylic acid suggests that the material is converted to an active compound more slowly than is the 5-adenylic acid.

SUMMARY

1. Adenosine, adenosine-3-phosphoric acid, adenosine-5-phosphoric acid, adenine, guanine, guanosine, guanylic acid, cytidine, cytidylic acid, xanthine, hypoxanthine, uracil, uridine, thymine, and ribose have been tested for their acute effects on the cardiovascular system.

2. Adenosine, adenosine-3-phosphoric acid, adenosine-5-phosphoric acid, and guanylic acid were the only ones which showed significant activity in the doses used.

3. These compounds lower the arterial pressure by direct peripheral vasodilation. In large doses transient sinus bradycardia is produced in atropinized or vagotomized dogs.

4. Bradycardia and a short period of decreased amplitude of contraction are produced in the perfused rabbit heart. The coronary flow is not significantly affected.

5. Some general relationships between chemical structure and dilator activity are presented.

REFERENCES

(1) Zipf, K., *Arch. exptl. Pathol. Pharmakol.*, **160**, 579 (1931).
(2) Zipf, K., *Klin. Wochschr.*, **1931**, 1521.
(3) Zipf, K., *Arch. exptl. Pathol. Pharmakol.*, **167**, 60 (1932).
(4) Zipf, K., and Wagenfeld, E., *ibid.*, **150**, 70, 91(1930).
(5) Bennett, D. W., and Drury, A. N., *J. Physiol.*, **72**, 228(1931).
(6) Kellaway, C. H., and Trethewie, E. R., *Australian J. Exptl. Biol. Med. Sci.*, **18**, 63(1940).

(7) Kellaway, C. H., and Trethewie, E. R., *ibid.*, **19**, 77 (1941).
(8) Kellaway, C. H., and Trethewie, E. R., *Quart. J. Exptl. Physiol.*, **30**, 121(1940).
(9) Kalckar, H. M., and Lowry, O. H., *Am. J. Physiol.*, **149**, 240(1947).
(10) Drury, A. N., *Physiol. Rev.*, **16**, 292(1936).
(11) Drury, A. N., and Szent-Gyorgyi, A., *J. Physiol.*, **68**, 213(1929).
(12) Wedd, A. M., and Drury, A. N., *J. Pharmacol. Exptl. Therap.*, **50**, 157(1934).
(13) Flömmer, O., *Arch. Exptl. Pathol. Pharmakol.*, **174**, 245(1934).
(14) Gillespie, J. H., *J. Physiol.*, **80**, 345(1934).
(15) Yutaka, J., *J. Oriental Med.*, **24**, 1185(1936).
(16) Robertson, I., and Shaw, F. H., *Australian J. Exptl. Biol. Med. Sci.*, **19**, 207(1941).
(17) Gropp, W., *Arch. Exptl. Pathol. Pharmakol.*, **199**, 216(1942).
(18) Hartman, W., *Z. Klin. Med.*, **121**, 424(1932).
(19) Ahlquist, R. P., THIS JOURNAL, **39**, 370(1950).
(20) Ahlquist, R. P., *J. Applied Physiology*, **5**, 48(1952).
(21) Anderson, F. F., and Craver, B. N., *J. Pharmacol. Exptl. Therap.*, **93**, 135(1948).
(22) Sherrod, T. R., and Louis, J., *ibid.*, **98**, 29(1950).

An Improved *in vivo* Method for Comparing the Constipative Effects of Drugs in Rabbits*

By WM. C. VAN ARSDEL, III, and NORMAN A. DAVID

A reliable and accurate estimate of narcotic drug effect on intestinal motility was obtained by making scybala counts at periods of one, three, five, seven, and, occasionally, fifteen hours after injection. The lowest dose of 0.05 mg./Kg. of morphine increased the scybala count during the first hour, but since the counts for the subsequent periods did not approach the control values, the over-all effect was that of a moderate degree of constipation. Morphine doses of 0.5 and 1 mg./Kg. decreased the scybala count up to the third hour after injection while the constipative effects of doses of 2 and 6 mg./Kg. were longer lasting. With 2 mg./Kg. little difference was noted in the suppression of the scybala count during the first seven hours whether food was allowed or removed. If, instead of graphing these results, the percentage of rabbits failing to defecate during an observation period is calculated, a more sensitive indicator is provided so that comparison of lower doses of constipating drugs on the intestinal tract may be made.

THE LABORATORY EVALUATION of drugs such as morphine which produce varying degrees of constipation has been unsatisfactory because of the few *in vivo* screening methods available. Neither intestinal strip nor balloon motility studies are directly applicable to the problem of constipation since these procedures measure only certain phases of the complex mechanisms involved in the normal progression and expulsion of intestinal contents. Of the methods available, Eddy's (1, 2) *in vivo* rabbit technique seems to offer the greatest promise. As originally designed, this method required that the subcutaneously administered dose of an opiate completely suppress intestinal evacuation in each of ten rabbits for one or more thirty-minute periods during the several hours after injection. With this definition, it was found that the minimal effective constipative dose of morphine was 6 mg./Kg. When Emerson (3) used this procedure to measure the laxative effect of prunes, he found that only a third of his control rabbits defecated during the observation period and since this was much lower than Eddy's control results, he attributed this difference to diet.

A modification of Eddy's method by Sato (4) showed that constipating drugs would significantly reduce the number of scybala passed by the rabbit each hour from 9 a. m. to 4 p. m. For this seven-hour period Sato's control rabbits regularly produced from 40 to 56 fecal pellets. With this technique the minimal effective constipative dose of morphine appeared to be 1 mg./Kg., but Krueger's (5) later tabulation of Sato's results indicate that 2 mg./Kg. is the dose. Sato also reported on the use of sympathectomized rabbits in this work, finding that morphine in dose ranges from 5 to 20 mg./Kg. exhibited little effect on scybala passing. Karr (6) used this modification to study the constipative effects of methadone and meperidine. Scott, *et al.* (7), reported that this method showed 5 mg./Kg. of morphine to be constipating while 2 mg./Kg. was stimulatory to the intestinal tract over an eight-hour observation period.

When we recently attempted to employ Sato's *in vivo* rabbit method for comparing the constipative effects of morphine and several new synthetic analgesics, a number of difficulties arose. It was found that the scybala counts made during the daytime were either very low, the majority of animals failing to defecate at times for periods of several hours, or the feces were passed in a soft

* Received November 12, 1953, from the Department of Pharmacology, University of Oregon Medical School, Portland, Ore.
Supported in part by a research grant for studies on new synthetic analgesics by Merck & Co., Rahway, N. J.

malformed mass which prevented accurate comparisons. Since normal values were practically impossible to obtain during the daytime and assuming that the rabbit (Lepus) still retains its nocturnal habits, we decided to carry out the observations from 5:00 p. m. on, at night. By this alteration in the time of observation and by suitably spacing the periods for making scybala counts after drug injection, we have been able to demonstrate with remarkable ability a dose relationship to intestinal effect for graded doses of morphine.

EXPERIMENTAL

Groups of sixteen rabbits were used for each observation. Each rabbit served as its own control in that two or three days previous to drug trial saline was injected following which control scybala counts were made. Injections of saline or morphine were made subcutaneously at 5:00 p. m. and the scybala collected and counted at one, three, five, seven, and fifteen hours thereafter. To permit graphic depiction of the results, the average number of scybala produced per hour by the group was calculated by dividing the average of the total number of scybala counted by the number of hours in the respective collection period. Drug administrations were spaced at least one week apart, or even longer, to reduce any possibility of drug cumulative effects.

RESULTS

Some drift of the average scybala count for the first-hour collection period was noticeable over the many months of study in the saline control rabbits due to variations in the hours of daylight. However, effects from seasonable variation appeared negligible since the changes from drug administration were out of this range of normal variability. It seemed feasible, therefore, to average all such control data together as shown in Fig. 1 for the "control, food *ad lib.*" graph.

Significant differences in scybala production may be caused by the presence or absence of food intake as shown by the two control graphs in Fig. 1. While our study does not permit outright separation of the influence of drug-induced anorexia from the constipative effect, the marked difference between the drug response and the effect of starvation (food removed at the time of drug injection) would suggest that anorexia represents a different type of response. For example, when a dose of 2 mg./Kg. of morphine was used, little difference was noted during the first seven hours in the scybala count whether food was allowed or removed.

With increases in the morphine dosage there was a graded decrease in the scybala count apparent for the first and third hour collection period. This average, as shown for the first hour periods in Fig. 1, masks the marked changes and variability in the count actually demonstrated by the individual rabbits given morphine. During the first hour the percentage of rabbits which do not defecate (see Table I) or which produce only one or two scybala is considerably increased. However, a few rabbits

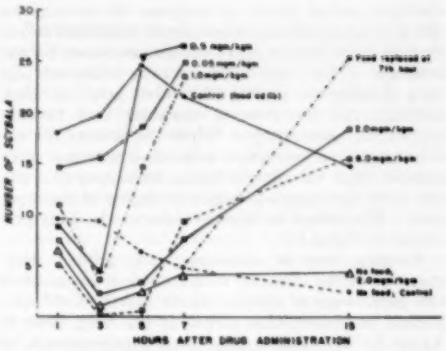


Fig. 1.—The effect of various doses of morphine on the rabbits' intestinal motility as measured by the production of scybala. The normal intestinal response with food *ad lib.* and under starvation conditions are also shown for comparison. It can be seen that there is a definite dose relationship in the response elicited.

show an immediate increase in scybala production or pass soft, unformed stools so that the first-hour average picture is misleading. This immediate defecatory response to morphine is well known and never unexpected in dogs and humans.

With the exception of the lowest dose of morphine (0.05 mg./Kg.) used, doses of 0.5, 1, 2, and 6 mg./Kg. decreased the scybala count for both the first and third hour after injection. Apparently the constipative effects of 0.5 and 1 mg./Kg. of morphine wore off soon after the third hour, being replaced by a considerably increased scybala count at the five-hour period. This effect may represent a residual stimulatory action of the drug on the intestinal tract or simply that the animal ate more food during this time. The graph shown in Fig. 1 for the first-hour count for 0.05 mg./Kg. of morphine does not depict the true situation since this determination was made late in June. The longer

TABLE I.—THE EFFECT OF GRADED DOSES OF MORPHINE IN SUPPRESSING SCYBALA PRODUCTION IN RABBITS

Drug	Dose, Mg./Kg.	No. of Observations	Per Cent of Rabbits Failing to Pass Scybala after Injection			
			0–1 Hr.	1–3 Hrs.	3–5 Hrs.	5–7 Hrs.
Saline	...	287	21	3	1	1
Saline[a]	...	48	29	10	12	22
Morphine sulfate	0.05	16	12.5	0	0	0
Morphine sulfate	0.5	16	50	6	0	0
Morphine sulfate	1	32	37	18	3	3
Morphine sulfate	2	32	56	50	34	3
Morphine sulfate	2	16	43	87	37	12
Morphine sulfate[a]	6	16	81	94	56	12

[a] Food removed at time of drug injection.

daylight period served to decrease the average for the first-hour control scybala count to around eleven instead of the higher figure of eighteen shown for the controls. Thus, there was actually increased scybala production during this first hour so that, initially, this low dose of morphine may be considered as stimulatory. While the counts for the subsequent periods show a gradual increase, they remain below the control values, indicating that this low dose also causes a moderate degree of constipation. This effect is more evident in the data presented in Table I.

Another way of measuring the graded dose-response of the intestine to morphine is to calculate the percentage of rabbits which failed to defecate during an observation period as has been done in Table I. We believe that such a presentation of the data obtained by following Eddy's procedure allows comparison of lower doses and serves as a more sensitive indicator of the effects of constipating drugs on the intestinal tract.

DISCUSSION

Previous *in vivo* methods of measuring the intestinal effect of drugs in the rabbit were subject to many biological limitations. The majority of rabbits in our colony produce formed scybala regularly only at night. The most sensitive indicator of drug effect must, then, be measurements taken at a time when formed scybala are consistently and regularly passed. Any tabulation for a period of time as long as eight hours after drug injection will usually obscure the time-response effect of the drug administered. Shorter observation periods show that the first effect of morphine is usually stimulatory with the formed scybala being expelled immediately after the drug injection. After this immediate stimulation there follows a period of constipation the duration of which increases as the dose is increased. This constipative effect can be measured only if observation periods are adequately spaced after the drug injection.

SUMMARY

1. By changing the time of observation from daytime to evening and by suitably spacing shorter periods for making scybala counts after administration of the drug, a reliable and accurate estimate of the effect of drugs on intestinal motility can be made.

2. Using such a modification, a dose-response relationship of morphine on rabbit intestinal motility can be demonstrated.

REFERENCES

(1) Eddy, N. B., *J. Pharmacol. Exptl. Therap.*, **45**, 339 (1932).
(2) Eddy, N. B., *ibid.*, **45**, 361(1932).
(3) Emerson, G. A., *Proc. Soc. Exptl. Biol. Med.*, **31**, 278 (1933).
(4) Sato, M., *Tôhoku J. Exptl. Med.*, **26**, 83(1935).
(5) Krueger, H., Eddy, N. B., and Sumwalt, M., "The Pharmacology of the Opium Alkaloids," Supplement No. 165, U. S. Public Health Reports, 1941, p. 484.
(6) Karr, N., *Fed. Proc.*, **6**, 343(1947).
(7) Scott, C. C., Chen, K. K., Kohlstedt, K. C., Robbins, R. B., and Israel, F. W., *J. Pharmacol. Exptl. Therap.*, **91**, 147(1947).

Extraction and Periodate Cleavage Studies of the Tannin Obtained from *Pinus caribaea*, Morelet*

By A. I. DINES† and F. W. BOPE‡

Alcoholic and aqueous extraction methods applied to *Pinus caribaea* are presented and compared. Through the measurement of a quantitative consumption of a specific glycol cleavage reagent, sodium metaperiodate, further evidence is presented in support of the suggestion that an alpha-glycol linkage exists in this natural phlobatannin molecule.

EARLY WORK on the structure of the phlobatannins by Russell and his co-workers suggested that these natural products were flavpinacol derivatives (1–5). The alpha-glycol linkage is characteristic of the flavpinacol structure. Bope and Gisvold in a preliminary phytochemical study of the tannin obtained from the slash pine tree, *Pinus caribaea*, Morelet, presented data showing the possibility of the existence of such a linkage in the structure of this phlobatannin molecule (6). To study further the possibility of the presence of the alpha-glycol linkage, oxidative cleavage reactions were conducted on a methylated derivative of the tannin, the specific alpha-glycol cleavage reagent sodium metaperiodate being used. The results were compared with those obtained by using lead tetraacetate as the cleavage reagent in a manner similar to that employed by Bope and Gisvold.

In addition to the oxidative cleavage reactions, a new method of extraction of the tannin was

* Received September 19, 1953, from the College of Pharmacy, The Ohio State University, Columbus, Ohio.
Based in part upon a thesis submitted by A. I. Dines to The Graduate School in partial fulfillment for the degree of Master of Science.
† Fellow of the Upjohn Company 1952–1953. Present address: The College of Pharmacy, The Ohio State University, Columbus, Ohio.
‡ Associate Professor of Pharmacy, The Ohio State University, Columbus, Ohio.

employed by which the tannin could be isolated without the use of heat and thereby minimize any possibilities of polymerization or decomposition. The method employed involved lyophylization of an aqueous extract of the bark. This method was compared with the alcoholic extraction method used by Bope and Gisvold.

EXPERIMENTAL

The inner bark of the slash pine tree, *Pinus caribaea*, Morelet, was used as a source of the tannin. The particular sample was collected in the Fall of 1949 through the courtesy of the Forest Service Division of the Department of Agriculture and was shipped from Olustee Experimental Forest, Olustee, Florida.

The bark was dried in a current of warm air upon receipt, later ground to a No. 20 powder, and then stored in an amber bottle at room temperature until used.

Preliminary Extraction.—The powdered bark was extracted in a Soxhlet for twenty-four hours with petroleum ether followed by extraction with ether for a similar period of time in order to remove those lipoids that are soluble in these solvents.

Extraction.—Two 1,000-Gm. portions of the defatted bark were macerated for three hours with 2,000 ml. of 95% alcohol and 2,000 ml. of water, respectively. The samples were percolated with alcohol and water. The first of the percolates gave a deep green color with $FeCl_3$ T. S. and a positive Molisch test. Percolation was continued until a negative $FeCl_3$ test was obtained. For the first sample, a total of 13.5 L. of 95% alcohol was required for complete extraction as indicated by a negative $FeCl_3$ test. However, a total of 24.5 L. of water was used to extract the tannin from the second sample. Percolation with water was stopped at 24.5 L., even though a slight positive $FeCl_3$ test was still being obtained.

The alcoholic percolate and the aqueous percolate were each divided into four equal portions and stored at 10°.

The alcohol was removed from one portion of the alcoholic percolate under reduced pressure with a minimum of heat from a steam bath. After drying in a vacuum desiccator over Drierite, the crude tannin weighed 70.5 Gm., or 28.2% of the defatted bark. This product was light brown, amorphous, and somewhat soluble in water. An aqueous solution of it gave a positive $FeCl_3$ test, positive Molisch test for sugar, and a positive phlobaphene test (7) when heated with a few drops of concentrated HCl. The crude tannin was stored in an amber bottle in the refrigerator.

The water was removed from one portion of the aqueous percolate by lyophylization in a commercial Stokes Freeze drying unit. The crude tannin obtained by this procedure was a fluffy, light brown, amorphous powder, very soluble in water, and weighed 63.5 Gm., or 25% of the defatted bark. An aqueous solution of this product gave the same positive qualitative tests as the crude tannin obtained from the alcoholic extract. This crude tannin was also stored in a refrigerator in an amber bottle.

Purification.—A slight modification of the tetrahydrofuran method employed by Bope and Gisvold (6) was used as a means of purification. The general procedure was as follows: 20 Gm. of the crude tannin was dissolved in a mixture of 200 ml. of freshly distilled tetrahydrofuran and 100 ml. of water to give a deep red solution. A small amount of the crude tannin remained undissolved. This solution was carefully decanted into a separatory funnel and an excess of anhydrous Na_2SO_4 was added to effect separation of the aqueous and tetrahydrofuran layers. After the separation was complete, the lower aqueous layer was drawn off. The remaining tetrahydrofuran solution of the tannin was then washed with 50-ml. portions of water to remove all traces of sugar from the tetrahydrofuran solution, as indicated by a negative Molisch test. In each case an excess of anhydrous Na_2SO_4 was added to effect separation of the two layers. Four such washings were required for the 20 Gm. of crude tannin extracted by alcohol, while five were required for the 20 Gm. of crude tannin extracted by water. In each case, the small amount of crude tannin remaining undissolved from the 20-Gm. sample was dissolved and washed free of sugars in a similar manner.

The tetrahydrofuran solutions of sugar-free tannin were dried overnight with Drierite. After removal of the Drierite by filtration, the solvent was removed by vacuum distillation. The purified product was then dried in a vacuum desiccator over Drierite.

The 20-Gm. portion of crude tannin from the alcoholic extraction yielded 13.0 Gm. of purified tannin. This represented 65% of the crude tannin, or 18.3% of the defatted bark. The 20-Gm. portion of crude tannin from the aqueous extraction yielded only 8.5 Gm., which represented 42.5% of the crude tannin, or 10.8% of the defatted bark.

The two purified tannins were amorphous and gave positive $FeCl_3$ tests as well as positive phlobaphene tests. However, they gave negative tests for sugar with Molisch reagent. They were stored in the refrigerator in amber bottles.

Methylation.—Two grams of the purified tannin obtained from the alcoholic extract of the defatted bark was dissolved in 30 ml. of methyl alcohol. This solution was shaken with an excess of sodium hydrosulfite to reduce any quinoid structures to free phenolic hydroxyls. The solution was then filtered and methylated immediately by the slow addition, with shaking, of 100 ml. of an ethereal solution of diazomethane. An excess of diazomethane (prepared from N-nitrosomethylurea) was used as indicated by a yellow color of the methylation mixture. During methylation a tan-colored precipitate was obtained. After standing overnight at 10°, the methylation mixture was filtered and the precipitate was washed with ether. The precipitate was amorphous and weighed 1.5 Gm. Concentration of the filtrate and dilution with water produced a cloudy solution which, after standing overnight, yielded an additional 0.3 Gm. of a tan amorphous product. After drying the combined methylated products in an Abderhalden drying pistol for six hours over P_2O_5, a methoxyl determination by the Clark modification of the Viebach and Schwappach procedure (8) showed 23.73% methoxyl.

The methylated tannin was remethylated with diazomethane to give a product that had a 27.55% methoxyl content.

Remethylation again in the same manner produced a buff-colored product which did not melt but decomposed above 230°. A methoxyl determination, following drying as before, showed 28.26% methoxyl. This value indicated the presence of three methoxyl groups for the $C_{15}H_{14}O_6$ catechin-type residue (calculated 28.02%) or six for Russell's flavpinacol-type structure with the empirical formula $C_{30}H_{26}O_6(OCH_3)_6$.

Periodate Oxidation.—The general procedure was as follows: A carefully weighed amount of the methylated tannin (0.150 Gm.) was dissolved in 20 ml. of glacial acetic acid, resulting in a yellow solution. Exactly 5 ml. of 0.04931 *M* (0.09862 *N*) sodium metaperiodate solution was added. The reaction mixture was then heated on a water bath at 50–55° for twenty-four hours. At the end of this period of time, the solution was a deep yellow-orange.

The solution was then neutralized by the slow addition of 100 ml. of 20% Na_2CO_3 solution followed by 1.5 Gm. of $NaHCO_3$. The resulting solution had a *p*H of about 6.5–7. To the neutralized solution, exactly 5 ml. of 0.500 *N* sodium arsenite solution and 5 ml. of approximately 1 *M* KI solution were added. After standing for five minutes, the excess sodium arsenite was determined by titration with 0.1014 *N* I_2 solution using starch as the indicator. Blanks containing only the reagents were carried out in a like manner. The titration indicated that 1.003 moles of $NaIO_4$ were consumed per mole of $C_{30}H_{26}O_6(OCH_3)_6$.

No attempt was made to isolate any of the cleavage products. However, after neutralization of the reaction mixtures, an orange-brown precipitate was present in each.

Lead Tetraacetate Oxidation.—In order to substantiate the periodate cleavage, an oxidative cleavage reaction was conducted on the methoxyl derivative using Criegee's reagent, lead tetraacetate.

The general procedure was as follows: a carefully weighed amount of the methylated tannin (0.150 Gm.) was dissolved in 20 ml. of glacial acetic acid, resulting in an orange-colored solution. Then 5 ml. of methyl alcohol was added as a catalyst followed by the addition of 20 ml. of 0.025 *M* (0.050 *N*) lead tetraacetate solution. The solution deepened in color. The reaction mixture was heated on a water bath at 50–55° under a reflux condenser fitted with a drying tube. After heating for twenty-four hours, the solution was a deep orange color. The excess lead tetraacetate was then titrated with standard sodium thiosulfate according to Criegee's procedure (9). Blanks were run on the reagents. The amount of $Pb(OAc)_4$ consumed was 1.011 moles per mole of methylated tannin, calculated on the basis of the empirical formula $C_{30}H_{26}O_6(OCH_3)_6$. Again at the end of the titration an orange precipitate was present in each reaction mixture.

SUMMARY

Although the aqueous extraction method resulted in a lighter colored, more water-soluble tannin, a larger volume of water was required for the extraction as compared to the amount of alcohol required. Furthermore, upon purification of the crude tannin extracted by water, a lower percentage of purified tannin resulted.

Upon methylation of the purified tannin with diazomethane, a methylated tannin was obtained which was in agreement with the empirical formula $C_{30}H_{26}O_6(OCH_3)_6$. When this methylated tannin was subjected to oxidative cleavage with sodium metaperiodate, 1.003 moles of $NaIO_4$ was consumed per mole of methylated tannin.

In order to support the sodium metaperiodate glycol cleavage of the methylated tannin, lead tetraacetate oxidative cleavage reactions were conducted on this derivative. Titrations indicated that 1.011 moles of lead tetraacetate was consumed per mole of the methylated tannin.

These positive quantitative glycol cleavage reactions support the suggestion that an alpha-glycol linkage exists within the natural phlobatannin molecule of *Pinus caribaea*, Morelet.

REFERENCES

(1) Russell, A., *J. Chem. Soc.*, **1934**, 218.
(2) Russell, A., and Todd, T., *ibid.*, **1934**, 1066.
(3) Russell, A., and Todd, T., *ibid.*, **1934**, 1506.
(4) Russell, A., Todd, J., and Wilson, C. L., *ibid.*, **1934**, 1940.
(5) Russell, A., and Clark, S. P., *J. Am. Chem. Soc.*, **61**, 2651(1939).
(6) Bope, F. W., and Gisvold, O., THIS JOURNAL, **38**, 192 (1949).
(7) Stahelin, C., and Hofstetter, J., *Ann.*, **51**, 63(1844).
(8) Clark, E. P., *J. Assoc. Offic. Agr. Chemists*, **15**, 136 (1932).
(9) Criegee, R., *Ber.*, **64**, 136(1932).

Protective Effect of Liver Residue on Immature Male Rats Fed Toxic Doses of Acetylsalicylic Acid*

By B. H. ERSHOFF, H. B. McWILLIAMS, and E. W. THURSTON

Administration of water-insoluble liver residue largely counteracted the growth retardation and reduction in thymus, prostate, and seminal vesicle weight of immature male rats fed toxic doses of acetylsalicylic acid. The protective factor(s) was distinct from any of the known B vitamins and was not present in significant amounts in purified casein.

IT IS BECOMING increasingly apparent that the effects obtained in an experimental animal following administration of drugs or related products are dependent on the nutritional state and the diets employed. In acute deficiencies of essential nutrients it is readily recognized that resulting abnormalities in cellular metabolism may profoundly affect response to certain drugs. What is less well recognized, however, is that drugs themselves may precipitate a deficiency state. Thyroid administration, for example, may induce a deficiency of thiamine, pyridoxine, pantothenic acid (1, 2), and vitamin B_{12} (3–5); while dicumarol, salicylates, and other drugs may precipitate a deficiency of vitamin K (6–8). Additional examples have been cited in a recent review (9).

In addition to the known nutrients, requirements for various unknown factors may also be increased following the administration of certain drugs. These "minor vitamins" are apparently dispensable under normal conditions, or requirements for them are so small that they may readily be met by amounts present in the diet, or through the synthetic activity of the intestinal flora, or the animals' own tissues. Following drug administration, however, requirements for these substances may be increased to the extent that deficiencies occur, manifested by retarded growth or tissue pathology and preventable by the administration in appropriate amounts of the missing nutrient (9, 10). Liver residue is a potent source of one or more of such nutrients.[1] Thus this liver fraction has been shown to counteract (at least in part) the deleterious effects of toxic doses of the following drugs: desiccated thyroid (11, 12), thyroxin, thyroglobulin, iodinated casein (13), Atabrine (14, 15), Promin (16), cortisone acetate (17), and succinylsulfathiazole (18). In the present communication, data are presented indicating that liver residue will also counteract (in part) the toxic effects of massive doses of acetylsalicylic acid in the rat.

PROCEDURE AND RESULTS

The basal ration employed in the present experiment consisted of sucrose, 60.5%; casein,[2] 24%; salt mixture,[3] 5%; cottonseed oil,[4] 8%; wheat germ oil,[5] 2%; acetylsalicylic acid,[6] 0.5%; and the following synthetic vitamins per kilogram of diet: thiamine hydrochloride, 20 mg.; riboflavin, 20 mg.; pyridoxine hydrochloride, 20 mg.; calcium pantothenate, 60 mg.; nicotinic acid, 60 mg.; ascorbic acid, 200 mg.; 2-methyl-naphthoquinone, 10 mg.; and choline chloride, 2 Gm. To each kilogram of diet were also added 4,000 U. S. P. units of vitamin A[7] and 400 U. S. P. units of vitamin D.[8] The vitamins were added in place of an equal amount of sucrose. In addition to the basal ration the following diets were also employed: (*a*) basal ration plus the following additional vitamin supplements per kilogram of diet: thiamine hydrochloride, 20 mg.; riboflavin, 20 mg.; pyridoxine hydrochloride, 20 mg.; calcium pantothenate, 60 mg.; nicotinic acid, 60 mg.; biotin, 5 mg.; folic acid, 10 mg.; *p*-aminobenzoic acid, 400 mg.; inositol, 800 mg.; and vitamin B_{12}, 150 micrograms; (*b*) basal ration plus 10% whole liver powder;[9] (*c*) basal ration plus 10% extracted liver residue;[10] and (*d*) basal ration plus 2.5% liver concentrate powder.[11] The vitamin supplements and liver fractions were added in place of an equal amount of sucrose. In addition to the above diets, a control ration was also tested, which consisted of the basal ration with acetylsalicylic acid omitted and replaced by an equal amount of sucrose.

---

* Received August 21, 1953, from the Emory W. Thurston Laboratories, Los Angeles, Calif., and the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles.

Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.

Communication 349 from the Department of Biochemistry and Nutrition, University of Southern California.

This investigation was supported in part by a grant from Merck & Co., Rahway, N. J.

[1] This fraction of liver consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

[2] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Wesson.

[5] VioBin.

[6] Acid Acetylsalicylic U. S. P. XIV Powder, Mallinckrodt.

[7] Crystalets, Chas. Pfizer & Co., New York.

[8] Hy-Dee Powder, Standard Brands, New York.

[9] Desiccated Liver N. F., Armour Laboratories, Chicago, Ill.

[10] Extracted Liver Residue, Wilson Laboratories, Chicago, Ill.

[11] Liver Concentrate Powder N. F., Armour Laboratories, Chicago, Ill.

Seventy-two male rats of the Long-Evans strain were selected at twenty-one to twenty-three days of age and at an average weight of 43.5 Gm. for the present experiment. The animals were placed in metal cages with raised screen bottoms, to prevent access to feces, and were fed the various diets *ad libitum* (12 animals per group). The diets were made up weekly and stored under refrigeration when not in use and the animals were fed on alternate days. All food not consumed forty-eight hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for sixty days or until death, whichever occurred sooner. The animals were autopsied after sixty days of feeding, and organ weights determined.

The results are summarized in Table I. The data were computed on the basis of the top ten animals in each group to minimize variations in average due to infection and atypical responses on the part of individual rats. Findings indicate that the effects of acetylsalicylic acid administration in the immature male rat are dependent on the diet employed. Rats fed the purified basal ration (which contained 0.5% acetylsalicylic acid) exhibited a significant retardation in growth and a reduction (both absolute and relative) in thymus, prostate, and seminal vesicle weights as compared to that observed in rats fed the same diet with acetylsalicylic acid omitted. These effects were largely counteracted by the administration of extracted liver residue and to a smaller extent by whole liver powder and liver concentrate. Supplements of the known B vitamins, however, had little, if any, effect. Both kidney and adrenal weights were increased when expressed in terms of milligrams per 100 Gm. body weight in all rats fed acetylsalicylic acid. Differences between the various groups, however, did not appear to be statistically significant. No difference in testes, ventricular, or liver weights was observed between any of the groups. The wet weight of the liver averaged approximately 4% of the total body weight (group averages ranged from 3.8 to 4.6% for the various groups); the per cent solids in the liver averaged approximately 30% (group averages ranged from 29.4 to 30.5%). The fat content of the liver for the various groups averaged from 8.0 to 11.1%; these differences were not statistically significant.

Subsequent experiments were conducted in which the gain in body weight of immature male rats fed the basal ration (containing 0.5% acetylsalicylic acid) was contrasted to that obtained in rats fed a similar diet supplemented with an additional 7% casein (added in place of an equal amount of sucrose). No significant difference between the two groups was noted over a sixty-day period. It would appear, therefore, that the protective effect of the liver residue-containing diet was not due to its higher content of protein *per se*.

In contrast to the findings in male rats, the following data indicate that liver residue had little, if any, protective effect in female rats fed toxic doses of acetylsalicylic acid. Thirty female rats of the Long-Evans strain were selected at 40–50 Gm. in weight and were fed the following diets (10 animals per group): (*a*) basal ration; (*b*) basal ration plus 10% liver residue; and (*c*) basal ration with acetylsalicylic acid omitted. After six weeks of feeding,

TABLE I.—COMPARATIVE EFFECTS OF B VITAMINS AND LIVER FRACTIONS ON THE BODY AND ORGAN WEIGHTS OF IMMATURE MALE RATS FED TOXIC DOSES OF ACETYLSALICYLIC ACID
(10 animals per group)[a]

Supplements Fed with Basal Ration	Acetylsalicylic Acid in Ration, %	Initial Body Weight, Gm.	Average Gain in Body Weight,[b,c] Gm.	Thymus Weight, Mg.	Thymus Weight, Mg./100 Gm. Body Weight	Prostate Weight, Mg.	Prostate Weight, Mg./100 Gm. Body Weight	Seminal Vesicles Weight, Mg.	Seminal Vesicles Weight, Mg./100 Gm. Body Weight	Testes Weight, Gm.	Adrenal Weight, Mg.	Adrenal Weight, Mg./100 Gm. Body Weight	Kidney Weight, Gm.	Kidney Weight, Gm./100 Gm. Body Weight	Ventricular Weight, Mg.	Ventricular Weight, Mg./100 Gm. Body Weight
None	0.5	43.4	141 ± 8.1	120	65	111	60	329	178	2.41	33	18	1.82	0.98	620	335
B vitamins	0.5	43.3	155 ± 10.4	180	90	132	66	370	186	2.56	35	18	1.99	1.00	720	377
2.5% Liver concentrate	0.5	43.3	187 ± 5.7	215	93	159	69	385	167	2.70	36	16	2.19	0.95	820	355
10% Desiccated liver	0.5	43.6	194 ± 8.9	288	121	153	64	515	216	2.63	43	18	2.41	1.01	770	324
10% Liver residue (Basal ration without acetylsalicylic acid)	0.5	43.4	214 ± 10.3	287	112	245	95	733	285	2.84	40	15	2.64	1.03	860	335
	0.0	43.4	228 ± 8.4	313	115	289	106	621	228	3.01	37	13	2.10	0.77	910	335

[a] Only nine rats survived on the basal ration and basal ration plus B vitamin groups. Data for these groups are based on the nine animals which survived in each series.
[b] Experimental period, sixty days.
[c] Including standard error of the mean calculated as follows: $\sqrt{\Sigma(d^2/n)}/\sqrt{n}$, where d is the deviation from the mean and n is the number of observations.

the average body weight for the three groups was as follows: basal ration, 141 Gm.; basal ration plus liver residue, 155 Gm.; and basal ration with acetylsalicylic acid omitted, 183 Gm. Nine out of the 10 rats in each group receiving acetylsalicylic acid and all control rats survived the six-week feeding period. The cause for the apparent sex difference is not readily apparent.

During the fourth week of feeding, total and differential white cell counts and total red cell counts were made on the tail blood of six male rats in both the basal ration and the basal ration without acetylsalicylic acid groups. Blood specimens were obtained after total body heating as previously described (19). The available data indicate that prolonged administration of ACTH (20) or cortisone acetate (17) causes a reversal in the differential count of the rat. Inasmuch as acetylsalicylic acid has been reported to stimulate pituitary-adrenal function (21, 22), changes in the leucocyte count were anticipated in rats fed acetylsalicylic acid in the present experiment comparable to those observed in animals administered ACTH or cortisone acetate. Such changes were not observed. No reversal in the differential count occurred in rats fed acetylsalicylic acid. The blood picture of rats fed the basal ration did not differ significantly from that of rats fed a similar diet with acetylsalicylic acid omitted. Findings in the two groups were, respectively, as follows: R. B. C., 5.8 million per mm.^{3} and 5.3 million per mm.^{3}; total W. B. C., 7,500 per mm.^{3} and 7,150 per mm.^{3}; per cent granulocytes, 25% and 27%; and lymphocytes per mm.^{3}, 5,600 and 5,200. It is apparent that the blood picture did not indicate adrenocortical hyperactivity as judged by the hematological changes following prolonged ACTH or cortisone acetate administration.

DISCUSSION

Present findings indicate that the administration of water-insoluble liver residue largely counteracted the growth retardation and the reduction in thymus, prostate, and seminal vesicle weight of immature male rats fed toxic doses of acetylsalicylic acid. The protective factor was apparently distinct from any of the known B vitamins and was not present in significant amounts in purified casein. The data available do not indicate whether the protective effect of liver residue was due to an unidentified factor (or factors) or to its content of other known nutrients. Since liver residue has also been found to contain a factor (or factors) distinct from any of the known B vitamins which counteracted the toxic effects of desiccated thyroid (11, 12), thyroxin, thyroglobulin, iodinated casein (13), Atabrine (14, 15), Promin (16), and cortisone acetate (17) administration in the rat, it is not unlikely that the protective effect in the present experiment was due to the same factor (or factors) which protected rats fed toxic doses of the drugs indicated above.

SUMMARY

1. Water-insoluble liver residue largely counteracted the growth retardation and reduction in thymus, prostate, and seminal vesicle weight of immature male rats fed toxic doses of acetylsalicylic acid.

2. The protective factor (s) was distinct from any of the known B vitamins and was not present in significant amounts in purified casein.

REFERENCES

(1) Drill, V. A., *Physiol. Rev.*, **23**, 355(1943).
(2) Drill, V. A., and Overman, R., *Am. J. Physiol.*, **135**, 474(1942).
(3) Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **70**, 40(1949).
(4) Emerson, G. A., *ibid.*, **70**, 392(1949).
(5) Betheil, J. J., and Lardy, H. A., *J. Nutrition*, **37**, 495(1949).
(6) Shapiro, S., Redish, M. H., and Campbell, H. A. *Proc. Soc. Exptl. Biol. Med.*, **52**, 12(1943).
(7) Shapiro, S., *J. Am. Med. Assoc.*, **125**, 546(1944).
(8) Collins, E. N., and Hoffman, A. D., *Cleveland Clinic Quart.*, **10**, 105(1943).
(9) Ershoff, B. H., "Nutrition Fronts in Public Health," Nutrition Symposium Series No. 3. The National Vitamin Foundation, Inc., New York, 1951.
(10) Ershoff, B. H., *Physiol. Revs.*, **28**, 107(1948).
(11) Ershoff, B. H., *Arch. Biochem.*, **15**, 365(1947).
(12) Ershoff, B. H., *Exptl. Med. Surg.*, **6**, 438(1948).
(13) Ershoff, B. H., and McWilliams, H. B., *Science*, **108**, 632(1948).
(14) Ershoff, B. H., *J. Nutrition*, **35**, 269(1948).
(15) Ershoff, B. H., *Arch. Biochem.*, **26**, 50(1950).
(16) Ershoff, B. H., and McWilliams, H. B., THIS JOURNAL, **38**, 274(1949).
(17) Ershoff, B. H., *Metabolism*, **2**, 59(1953).
(18) Bosshardt, D. K., and Huff, J. W., *J. Nutrition*, **50**, 117(1953).
(19) Ershoff, B. H., and Gaines, J. G., *Science*, **118**, 20 (1953).
(20) Asling, C. W., Reinhardt, W. O., and Li, C. H., *Endocrinology*, **48**, 534(1951).
(21) Hallman, H. F., *J. Clin. Endocrinol. and Metabolism*, **12**, 454(1952).
(22) Pasqualini, R. Q., *ibid.*, **12**, 1246(1952).

Stability of Some Bactericidal Chlorine-Liberating Compounds in Special Ointment Bases*

By ABRAHAM TAUB,† FANCHON HART,‡ and STANLEY KASSIMIR§

A comparison is made of the stability of three chlorine-liberating compounds: dichloramine-T, chloroazodin, and trichloromelamine, in four anhydrous ointment vehicles of relatively nonreactive character—white petrolatum, a silicone gel, a halogenated hydrocarbon, and a mineral oil gelled by polyethylene. Two dispersing agents—glyceryl triacetate and polyoxyethylene glycol 1000 dilaurate—were employed. When dispersed in glyceryl triacetate, chloroazodin appears most stable in the halogenated hydrocarbon and the gelled mineral oil bases, and only slightly less stable in the silicone gel. Trichloromelamine and dichloramine-T appear most stable in the halogenated hydrocarbon base. Of the chemically stable ointments, only those containing chloroazodin showed significant zones of inhibition in the filter disk method when tested against *M. pyogenes* var. *aureus*, *S. typhosa*, and *E. coli*.

ALTHOUGH chlorine-liberating compounds are generally effective antiseptics, their poor solubility in fatty vehicles, and their marked reactivity in the presence of moisture or most organic matter has limited their utilization in ointment form. Dichloramine-T, one of the first of the organic chlorine-liberating antiseptics developed, and more stable than the inorganic hypochlorites, has been utilized in ointment or topical form through the medium of a special solvent, chlorcosane (1), a chlorinated paraffin. However, its shelf life has not proved serviceable and this solvent is no longer available.

Chloroazodin,[1] which because of its lower oxidation potential (2) has been found to react more slowly with organic matter than previously investigated chlorine-liberating compounds (3), remains stable for longer periods of time. However, its stability and bactericidal effectiveness in its presently available ointment form are not as great as when it is present in simple triacetin solution.

More recently a new chlorine-liberating compound of the chloramine type, trichloromelamine[1] has been introduced (4) as a germicidal rinse. It has a very high "available" chlorine content (92%) and good stability in the dry form. No information on its usefulness in ointments has been published.

It is the purpose of the present study to determine whether these three compounds, all containing positively charged chlorine, can be formulated into stable antiseptic ointment forms.

METHOD

The ointment vehicles selected represented substances believed to be of relatively low reactivity toward chlorine:

(*a*) Petrolatum U. S. P., superwhite, highly refined grade. (Penn-Drake Petroleum Products, Butler, Pa.)

(*b*) Jelene 50 W., a bland mineral oil gelled by special process with polyethylene of average mol. wt. 1300 (5–7). (Research Products Corp., Madison, Wis.)

(*c*) Silicone No. 7 Compound, a nonmelting gel retaining its semisolid consistency from −40 to 200°, representing dimethyl polysiloxane fluid, a nontoxic silicone oil (8, 9), gelled with silica. (Dow Corning Corp., Midland, Mich.)

(*d*) Halocarb, a nonmelting polymerized chlorotrifluoroethylene, average mol. wt. 900, of petroleum jelly consistency. (Halocarbon Products Corp., North Bergen, N. J.) This is a stopcock lubricant, represented as being completely inert toward oxidizing agents. In laboratory and industrial use it has proved nonirritant to the skin, but no physiological study has been published on it to date.

Since the chlorine-liberating compounds are not soluble in these ointment vehicles, it was necessary to find suitable solubilizing or dispersing agents of low reactivity toward chlorine. After screening a number of substances, two were selected for further experimentation: polyoxyethylene glycol 1000 dilaurate, and glyceryl triacetate. Both are relatively good solvents for all three compounds. Later in the study another solubilizing agent came to our attention, acetyl triethyl citrate. Though reported (10) as a good solvent for dichloramine-T and chloroazodin, it was not found in the present study to have any advantages over glyceryl triacetate; the latter was in fact the better solvent for the trichloromelamine.

To determine whether a surface-active agent would increase the effectiveness of penetration in microbiological tests, a fifth ointment base was prepared containing 1% dioctyl sodium sulfosuccinate N. F. in petrolatum.

The ointments were prepared in glass containers by first dispersing or dissolving the chlorine-liberating agent in glyceryl triacetate or in melted (40°) polyoxyethylene dilaurate, then thoroughly mixing

* Received August 21, 1953, from Columbia University, College of Pharmacy, New York.
Presented to the Scientific Section, A. Ph. A., Salt Lake City meeting, August, 1953.
† Professor of Pharmaceutical Chemistry, Columbia University, College of Pharmacy.
‡ Professor of Biology, Columbia University, College of Pharmacy.
§ Assistant in Chemistry.
[1] Marketed by Wallace & Tiernan Products, Inc., Belleville, N. J.

with about 10% of the ointment vehicle followed by the balance of the vehicle. In all experiments 0.1% of chlorine-liberating agent and 1% of dispersing agent were employed. The ointments were stored in well-stoppered amber ointment jars at 25° ± 3° and at 40°.

Ointments were assayed by dissolving 4-Gm. samples in 50 ml. of boiling chloroform; 50 ml. of distilled water, 20 ml. of glacial acetic acid, and 0.2 Gm. potassium iodide were added in the order given. The liberated iodine was titrated with 0.02 *N* sodium thiosulfate after a thirty-minute storage in the dark with occasional agitation. Starch T. S. was used as indicator.

Results for the chemical stability of the ointments under storage at room temperature and at 40° are given in Tables I, II, and III.

TABLE I.—STABILITY OF CHLOROAZODIN DISPERSED BY POLYOXYETHYLENE GLYCOL 1000 DILAURATE IN OINTMENT BASES

Ointment Base	Percentage Decomposition 3 Mo., 25° ± 3	Percentage Decomposition 6 Wks., 40°
Petrolatum	5.9	42.0
Petrolatum with dioctyl sodium sulfosuccinate	0.4	36.8
Silicone No. 7 Compound	5.2	100.0
Jelene 50 W.	0.1	29.0
Halocarb	19.6	75.9

TABLE II.—STABILITY OF CHLOROAZODIN DISPERSED BY GLYCERYL TRIACETATE IN OINTMENT BASES

Ointment Base	Percentage Decomposition 5 Mo., 25°±3	Percentage Decomposition 3 Mo., 40°	Percentage Decomposition 5 Mo., 40°
Petrolatum	0.2	10.0	45.2
Petrolatum with dioctyl sodium sulfosuccinate	0.2	6.0	30.3
Silicone No. 7 Compound	0.1	4.3	13.7
Jelene 50 W.	0	1.8	2.3
Halocarb	0	0	2.4

TABLE III.—STABILITY OF TRICHLOROMELAMINE DISPERSED BY GLYCERYL TRIACETATE IN OINTMENT BASES

Ointment Base	Percentage Decomposition 5 Mo., 25°±3	Percentage Decomposition 3 Mo., 40°	Percentage Decomposition 5 Mo., 40°
Petrolatum	63.2	86.7	100.0
Petrolatum with dioctyl sodium sulfosuccinate	87.9	100.0	100.0
Silicone No. 7 Compound	35.5	22.0	46.9
Jelene 50 W.	40.0	48.5	55.8
Halocarb	0	1.3	1.2

From the results it appears that with respect to chemical stability most of the bases would prove suitable for the prescription compounding of chloroazodin. However, for longer shelf life this series would not prove appropriate. A further check on the dispersing agent showed that when polyoxyethylene glycol 1000 dilaurate was used as the ointment vehicle (99.9%) for the chloroazodin, it provided a stable base for a period of three weeks at room temperature, but resulted in 100% decomposition after six weeks at 40°. It is of interest to note that both dichloramine-T and trichloromelamine in this series of bases showed 100% decomposition within three weeks at room temperature. Therefore, it was decided to limit further work in this study to the use of glyceryl triacetate as the dispersing agent since the dilaurate ester even at a 1% concentration had a significant deteriorating effect.

No table is given for dichloramine-T since with the exception of Halocarb all of the ointment bases caused its complete decomposition after five months' storage at 25° and at 40°. Instantaneous liberation and complete loss of chlorine occurred when dichloramine-T was mixed with Silicone No. 7 Compound. Halocarb provided a fairly stable vehicle for dichloramine-T, the decomposition being but 4.3% at 25° and 6.7% at 40° after five months' storage.

The results of Tables II and III indicate that the nature of the chlorine-liberating agent, as well as the vehicle, influences the rate of decomposition. Chloroazodin is the most stable agent, while halocarb provides the most stable base. Jelene is almost as good, and Silicone No. 7 compound provides nearly as stable a base for the chloroazodin. Trichloromelamine is stable only in halocarb.

A further check on the dispersing agent, glyceryl triacetate, showed that all three chlorine-liberating agents are stable in this vehicle, less than 1% loss of chlorine occurring at 25° storage for five months.

The above results reflect only the chemical stability of the ointments. To determine their *in vitro* efficacy as antiseptics, zones of inhibition against organisms on nutrient agar plates were determined, using the paper disc assay procedure (11). Three organisms were employed: *Micrococcus pyogenes* var. *aureus* M-209, *Salmonella typhosa* (Hopkins Strain), and *Escherichia coli*. The paper discs (12.7 mm.) covered with ointment samples were placed upon the plates containing the respective organisms and zones of inhibition determined after twenty-four hours' incubation at 37°. Determinations for ointments initially prepared and after storage periods at room temperature, and at 40° are shown in Tables IV and V. Experimental results in the presence of 10% horse serum are shown in Table VI. Controls, containing dispersing agent, surface-active agent, and ointment bases, without the chlorine-liberating agents, exhibited no zones of inhibition.

The data in the above tables are limited to chloroazodin since neither trichloromelamine nor dichloramine-T exhibited zones of inhibition in any of the ointment bases initially. In control samples containing 0.1% of chlorine-liberating agent in pure glyceryl triacetate, trichloromelamine and dichloramine-T gave, respectively, 8 mm. and 5 mm. zones of inhibition. None of the ointment bases tested, therefore, can be considered as suitable vehicles for the diffusion of these two antiseptic agents.

Chloroazodin when dispersed by polyoxyethylene glycol 1000 dilaurate gave good initial inhibition in the Silicone, Jelene and Halocarb bases, but showed reduction or loss of activity on storage, paralleling the chemical stability results.

TABLE IV.—ZONES OF INHIBITION: CHLOROAZODIN DISPERSED BY POLYOXYETHYLENE GLYCOL 1000 DILAURATE IN OINTMENT BASES

Organism	Storage	Ointment Bases (Width of Zone in Mm.) Petrolatum	Petrolatum and S. A. Agent	Silicone No. 7 Compound	Jelene 50 W.	Halocarb
M. aureus	Initial	0	0	6	5	6
	3 mo., 25° ± 3	0	0	6	4	8
	6 wks., 40°	0	0	0	2	0
S. typhosa	Initial	0	0	8	4	17
	3 mo., 25° ± 3	0	0	7	4	16
	6 wks., 40°	0	0	0	2	5
E. coli	Initial	0	0	6	2	5
	3 mo., 25° ± 3	0	0	6	2	0
	6 wks., 40°	0	0	0	1	0

TABLE V.—ZONES OF INHIBITION: CHLOROAZODIN DISPERSED BY GLYCERYL TRIACETATE IN OINTMENT BASES

Organism	Storage	Ointment Bases (Width of Zone in Mm.) Petrolatum	Petrolatum and S. A. Agent	Silicone No. 7 Compound	Jelene 50 W.	Halocarb
M. aureus	Initial	0	2	5	3	11
	5 mo., 25° ± 3	0	2	5	3	10
	3 mo., 40°	0	2	5	3	12
	5 mo., 40°	0	1	5	3	8
S. typhosa	Initial	0	3	8	8	9
	5 mo., 25° ± 3	0	3	7	3	10
	3 mo., 40°	0	3	7	4	7
	5 mo., 40°	0	2	6	4	8
E. coli	Initial	0	1	3	2	4
	5 mo., 25° ± 3	0	1	4	2	2
	3 mo., 40°	0	0	4	2	4
	5 mo., 40°	0	0	4	2	4

TABLE VI.—ZONES OF INHIBITION IN PRESENCE OF SERUM: CHLOROAZODIN DISPERSED BY GLYCERYL TRIACETATE IN OINTMENT BASES

Organism	Storage	Ointment Bases (Width of Zone in Mm.) Petrolatum	Petrolatum and S. A. Agent	Silicone No. 7 Compound	Jelene 50 W.	Halocarb
M. aureus	Initial	0	0	2	2	3
	5 mo., 25° ± 3	0	0	1	1	2
	5 mo., 40°	0	0	1	1	1
S. typhosa	Initial	0	1	2	2	5
	5 mo., 25° ± 3	0	1	1	1	2
	5 mo., 40°	0	0	1	1	2
E. coli	Initial	0	2	2	2	2
	5 mo., 25° ± 3	0	2	2	2	2
	5 mo., 40°	0	0	2	1	2

With glyceryl triacetate as the dispersing agent, zones of inhibition were maintained on storage in the above three bases. There was a slight inhibition in petrolatum containing dioctyl sodium sulfosuccinate as a surface-active agent. Petrolatum without this agent did not permit a satisfactory diffusion of the chlorine liberating agent even in the presence of glyceryl triacetate.

The antiseptic activity of chlorine is known to be reduced in the presence of much organic matter. This is borne out by the results in the presence of 10% horse serum (Table VI). Halocarb appears to be slightly superior to Silicone and Jelene, while all three retain a slight activity upon storage.

The relatively small zones of inhibition reported in these tables may be attributed to the low concentration of active agent, 1:1,000, a concentration which, however, was selected to avoid irritation on topical application.

CONCLUSIONS

1. Of three organic chlorine-liberating antiseptics tested, only chloroazodin maintains its effectiveness in ointment form.

2. A dispersing agent, glyceryl triacetate, is required to permit uniform diffusion of the active

agent throughout the anhydrous ointment vehicle.

3. Halocarb, silicone no. 7 compound, and Jelene 50 W appear to provide stable ointment vehicles for chloroazodin since the antiseptic properties of the latter are maintained during accelerated storage periods of five months at 40°. The presence of considerable organic matter reduces but does not vitiate this activity.

4. Petrolatum does not appear to be a suitable vehicle for chloroazodin.

REFERENCES

(1) Dakin, H. D., and Dunham, E. K., *Pharm. J.*, **100**, 82(1918).
(2) Schmelkes, F. C., and Marks, H. C., *J. Am. Chem. Soc.*, **56**, 1610(1934).
(3) Guiteras, A. F., and Schmelkes, F. C., *J. Biol. Chem.*, **107**, 235(1940).
(4) *Chemical Week*, **71**, 47(1952).
(5) U. S. Pat. 2,628,187.
(6) Foster, S., Wurster, D. E., Higuchi, T., and Busse, L. W., THIS JOURNAL, **40**, 123(1951).
(7) Jones, E. R., and Lewicki, B., *ibid.*, **40**, 409(1951).
(8) Plein, J. B., and Plein, E. M., *ibid.*, **42**, 79 (1953).
(9) Lesser, M. A., *Drug & Cosmetic Ind.*, **72**, 616(1953).
(10) U. S. Pat. 2,618,584.
(11) Hoffman, H., and Phillips, C. R., *Ann. N. Y. Acad. Sci.*, **53**, 59(1950).

The Preparation and Evaluation of Tangerine Peel Tincture and Tangerine Syrup*

By WILLIAM J. SHEFFIELD† and HERMAN O. THOMPSON‡

A tangerine peel tincture was prepared by maceration of the whole peel from the fresh fruit. In the determination of the oil content of the tinctures, an adaption of the A. O. A. C. precipitation method appeared to have a greater general accuracy than the polarization method. A tangerine syrup was prepared from the tincture, using light magnesium carbonate as the filtering agent. Organoleptic tests indicated that tangerine syrup compared favorably in taste preference with five other vehicles which had ranked high in taste preference tests conducted by other workers. Tangerine syrup also appeared to have a high value in disguising the salty taste of ammonium chloride and a mediocre value in disguising the bitter taste of quinine hydrochloride.

THE TANGERINE is a variety of the king orange, *Citrus nobilis*, which was first introduced into California from China in 1880 (1) and later into Florida. The fruit of the king orange has a poor appearance and has never been popular, but the popularity of the reddish-orange tangerine, var. *deliciosa*, has increased steadily through the years. In Florida, two to six million boxes of tangerines are produced annually. As much as one-third of the crop cannot be shipped because of the restriction on size and grade (2), therefore availability of the fruit for by-product outlets is assured.

Tangerine oil is recovered commercially and is sold in a limited market. It has a sweet, distinctive flavor and is well adapted for use in beverages and confections. It is used to some extent in Eau de Cologne. Analysis of Florida machine-pressed tangerine oils during the 1946 season varied within the following limits: sp. gr., 0.850 to 0.856; $[\alpha]\ ^{25}_{D}$°, 91 to 95 degrees; $n\ ^{25}_{D}$°, 1.4730 to 1.4743; aldehydes as decyl aldehyde, 0.6 to 0.0 per cent (3). The high optical rotation is evidently caused by the lower content of *l*-limonene, as Nelson (4) found that *d*-limonene was present in amounts as high as 95 per cent. He also found small amounts of octyl aldehyde, linalool, citronellol, citral, and sesquiterpenes. The ester content was low and no methyl anthranilate was detected.

* Received October 24, 1951, from the University of North Carolina School of Pharmacy, Chapel Hill, N. C.
Presented to the Scientific Section, A. PH. A., Atlantic City meeting, August, 1950.
Abstracted from the thesis submitted by William J. Sheffield to the Graduate Faculty of the University of North Carolina in partial fulfillment of the requirements for the degree of Master of Science.
† North Carolina Pharmaceutical Research Foundation Fellow. Present address: University of Texas.
‡ Professor of Pharmacy, School of Pharmacy, University of North Carolina.

EXPERIMENTAL

Preparation of Tangerine Peel Tincture.—Nine samples of tangerine peel tinctures were prepared by a method similar to that of making sweet orange peel tincture, U. S. P. XIV (5). The first tincture (*A*) was made using the freshly grated outer peel and was macerated, with frequent shaking, for five days. It proved difficult to grate the outer peel, because of its thinness and loose attachment to the pulp. Inasmuch as the peel is easily removed and does not contain a bitter inner rind, grating of the outer peel did not appear feasible. Therefore, seven other tinctures were prepared by maceration of the whole peel which was broken up into small pieces. All tinctures were kept in tightly stoppered flasks at room temperature, in the dark, and were shaken twice a day for three minutes in a mechanical shaker. Tincture No. 1 was macerated for one day; No. 2 for two days; No. 3 for three days; No. 4 for four days; No. 5 for five days; No. 6 for seven days, and No. 7 for nine days. Tincture No. 8 was prepared in the same manner but was macerated for one year with only occasional shaking. After filtering, all the tinctures were a brilliant orange color and had a characteristic tangerine odor.

In order to ascertain the optimum length of maceration time, the oil content of each of the sample tinctures was determined. For this purpose, two methods, official in *Official and Tentative Methods of Analysis* of the Association of Official Agricultural Chemists, were used and proved satisfactory when modified for tangerine oil. The precipitation method (6) was followed as given except for the correction used to compensate for the solubility of the oil. To determine the solubility correction for tangerine oil, known percentage strength tinctures were made using tangerine oil[1] and alcohol. Ten tinctures ranging in oil content from 0.5 to 5.0% v/v were used. A series of eight samples of the same strength was run simultaneously, and the percentage value shown by the majority of the samples was used as the final percentage of the oil present.

In the polarization method (6), a new constant suitable for tangerine oil had to be determined. Five tinctures of known oil percentage were prepared containing 0.5%, 1.0%, 1.5%, 2.0% of tangerine oil by volume. The specific rotation was determined for each sample, and the angular rotation converted to degrees Ventzke. The constant for tangerine oil was obtained by dividing the degrees Ventzke by the known percentage of oil. The results obtained by both methods are shown in Table I.

The solubility correction factor of tangerine oil in the precipitation method was 0.3% for tinctures containing more than 1.0% of oil and 0.2% for those containing less than 1.0% of oil. The constant necessary for tangerine oil in the polarization method was 4.80.

The oil content of all tinctures prepared by maceration was then determined by the precipitation and/or polarization methods. Results of these determinations are shown in Table II.

The two methods agreed quite closely in most instances, although the results obtained for the tinctures with a maceration time of five and nine days showed an unusual and unexplained variation. The results also varied widely on the tincture which had a maceration time of one year. However, this could be explained by the findings of Netz (7) who stated that the specific rotation of lemon extract varied between different samples of the same kind of oil, and also that it decreased markedly as the age of the oil increased. The polarization method, therefore, would appear to be satisfactory only for tinctures of recent manufacture, while the precipitation method would be preferable for general use because of its greater overall accuracy.

Table II shows that, after three days, the length of maceration time of the whole peel did not appreciably change the oil content of the tincture. The tincture prepared by maceration of the grated outer peel contained the largest percentage of oil. However, this advantage would not seem to outweigh greatly the difficulty presented in its preparation.

The alcohol determination of tangerine peel tincture was carried out in essentially the same manner as given in Woodman's *Food Analysis* (8) except that a 25-cc. sample was used and the per cent of alcohol by volume was multiplied by 4 to obtain the percentage of the original tincture.

The alcohol content of the seven tinctures with maceration periods of one through nine days showed

TABLE I.—DETERMINATION OF CORRECTION FACTORS AND THE CONSTANT FOR TANGERINE OIL IN ASSAY OF TANGERINE PEEL TINCTURE

Known Per Cent of Oil	Determined Per Cent of Oil by Precipitation	Solubility Correction Factor	Degrees Ventzke by Polarization	Constant for Tangerine Oil
5.0	4.70	0.30	...	...
4.5	4.20	0.30	...	...
4.0	3.70	0.30	...	...
3.5	3.20	0.30	...	...
3.0	2.70	0.30	...	...
2.5	2.20	0.30	11.77	4.71
2.0	1.70	0.30	9.39	4.70
1.5	1.20	0.30	7.10	4.73
1.0	0.70	0.30	4.85	4.85
0.5	0.30	0.20	2.51	5.02
			Average	4.80

TABLE II.—ASSAY OF TANGERINE PEEL TINCTURES FOR OIL CONTENT

Maceration Time in Days	Percentage of Oil Found by Precipitation Method	Percentage of Oil Found by Polarization Method[a]	Percentage Difference in Oil Content
1	1.60	1.64	0.04
2	1.70	1.66	0.04
3	1.89	1.89	0.00
4	1.90	1.92	0.02
5	1.71	1.90	0.19
7	1.95	1.92	0.03
9	1.85	1.66	0.19
364	2.00	0.79	1.21
5[b]	2.80	...	...

[a] The constant 4.9 was used to determine these results as it gave figures more comparable to the results of the precipitation method than did the actually determined 4.8.

[b] Tincture *A* prepared from grated outer peel.

[1] Tangerine oil furnished by Warren E. Burns, Inc., Chicago, Ill.

a range of 68.20 to 69.72%. The optimum maceration period of three days yielded a tincture which contained 68.56% alcohol.

Preparation of Tangerine Syrup.—Three different samples of tangerine syrup were prepared by the same method as that used in the preparation of orange syrup, U. S. P. XIV (9). The only change made was in the amount of tincture used. Syrup *A* contained 50 cc., syrup *B* 100 cc., and syrup *C* 150 cc. of tangerine peel tincture. Syrup *A* had a delicate but characteristic odor and flavor and was nearly colorless. Syrup *B* was a light yellow color with the tangerine odor and flavor more pronounced. The color of syrup *C* was slightly deeper than that of syrup *B*, but the flavor was strong and left a rather disagreeable aftertaste. Therefore, 100 cc. of tangerine peel tincture per 1,000 cc. of syrup was used in the experimental vehicle, and, due to the slightly decreased solubility, 800 Gm. of sucrose.

The question arose as to the efficacy of talc as the filtering agent in this preparation. In filtering the aqueous-alcoholic solution, a considerable amount of coloring matter was removed from the tincture by talc, and it was necessary to return several portions of the filtrate through the filter before the solution filtered clear. It was desirable to reduce the filtration time of such an aromatic solution and also to retain as much of the color as possible. Therefore, filtration tests were conducted in which six different agents were used and their efficiency compared. The results of these tests are shown in Table III.

The volume of solution filtered with each filtering agent was the amount necessary to make 1,000 cc. of the finished syrup or 450 cc. Different amounts of each agent were first tested, and the number of grams shown in Table III represented the least amount which was most efficient as a filtering aid. In some cases the use of a larger amount of the filtering agent increased its filtering efficiency with respect to clarity of the filtrate but decreased its value with respect to bulk, absorption of solution, length of filtration time, and absorption of color.

In the filtration tests the citric acid was triturated with the tincture and filtering agent, water was added slowly with continued trituration, and the suspension filtered through a dry, plaited filter paper. Table III shows that reactions occurred between some of the filtering agents and the citric acid and produced increased *p*H values of the solutions. As the palatability and disguising value of the syrup were enhanced by the tangy, acidulous taste, it was necessary to change the filtration procedure in order to preserve the definite acid *p*H of the desired product. This was accomplished when the addition of the citric acid was made after the solution had been filtered clear. This procedure produced solutions with a *p*H comparable to that of the solution filtered with talc.

With respect to the amount of material used, the length of time of filtration, the *p*H of the final product, and the satisfactory color of the syrup, light magnesium carbonate was the filtering agent of choice, with the provision that the addition of the citric acid be made after the aqueous-alcoholic solution had been filtered clear. The alcoholic content of this syrup was 6.12% by volume, and the specific gravity was 1.280 at 25°.

*p*H determinations of tangerine syrups prepared with talc, calcium phosphate, and magnesium carbonate as the filtering agents showed no significant changes after eight months' storage in clear glass containers, at room temperature, and in the presence of light. Deterioration of these syrups after this length of time was very slight. There was some loss of color, slight evidence of bacterial or mold growth, and though the syrups were still palatable, there was a slight disagreeable aftertaste.

Organoleptic Tests.—In order to evaluate tangerine syrup in terms of palatability alone, organoleptic tests were conducted in which the flavor preference of tangerine syrup and five other flavoring syrups was determined. The tests were conducted in the same manner as that developed by Wright (10). Thirty subjects participated in each test and 75 different subjects took part in all the tests. Because Purdum (11) found that the taste of some full-strength syrups persisted even after several washings with distilled water, all vehicular solutions contained 10% by volume of the syrup in distilled water.

Since a total of 6 syrups was used, there were 15 different combinations of 4 which had to be tested in order to compare each syrup with every other syrup. The results were expressed as a percentage

TABLE III.—THE EFFICIENCY OF FILTERING AGENTS IN THE PREPARATION OF TANGERINE SYRUP

Filtering Agent	Amount of Filtering Agent, Gm.	Filtration Time, Min.	*p*H of Solution Filtered with Citric Acid	*p*H of Solution (Citric Acid Added after Filtration)	*p*H of Final Syrup
Talc[a]	25	60	2.71	2.75	2.75
Calcium phosphate[b] (dibasic)	125	30	3.25	2.58	2.61
Magnesium carbonate[c] (light)	20	20	7.50	2.55	2.58
Magnesium carbonate[d] (heavy)	50	30	4.55	2.77	2.79
Pulped filter paper[e]	25	..	...	...	...
Infusorial earth[f] (purified)	25	60	2.63	2.58	...

[a] 100-cc. portions of filtrate returned through filter six or seven times.
[b] Filtrate clear after a few cc.
[c] Filtrate clear immediately.
[d] 100-cc. portions of filtrate returned through filter three times.
[e] Did not filter clear.
[f] Filtrate clear after first few cc.

TABLE IV.—TASTE PREFERENCE OF SIX SYRUPS TESTED IN SETS OF FOUR

Vehicle	Mean Per Cent Preference
Orange syrup (U. S. P. XIII)	63
Tangerine syrup	60
Raspberry syrup[a]	52
Cacao syrup (N. F. VIII)	45
Cinnamon syrup (N. F. VIII)	42
Cherry syrup (N. F. VIII)	38

[a] Prepared by N. F. VIII method from the juice of canned raspberries.

of a perfect first choice, and the average per cent score for each syrup is shown in Table IV.

The results in Table IV indicated that tangerine syrup was of promise with respect to flavor alone because it was second in taste preference in a group of vehicles, four of which were rated as the most preferred by Wright (10).

The value of tangerine syrup as a disguising agent for drugs was determined by duplicating the procedure of Purdum (11), which does not take into consideration the palatability of the vehicle but is an accurate estimation of its disguising properties. The threshold taste for the drug in an aqueous solution is compared with the threshold taste for the drug in a solution of the vehicle. The difference between the two thresholds is expressed numerically as the disguising potential of the vehicle for the drug used. Generally, in a group of vehicles, the higher the mean disguising potential, the greater the disguising value for the drug used.

Ammonium chloride was used as a drug having a salty taste, and quinine hydrochloride as a drug having a bitter taste. The concentrations of both drugs in water and in the vehicles are shown in Table V.

The normality of ammonium chloride in the 10% v/v tangerine syrup was of higher concentration than the corresponding aqueous solution. The purpose of this was to reduce the number of solutions necessary for the test. When the results were calculated, the number recorded as the threshold in the vehicular solutions was increased by one. In the case of quinine hydrochloride, tube No. 1 of the vehicular solutions corresponds to tube No. 4 of the aqueous solutions. When the results of tnis test were calculated, the number recorded as the threshold in the vehicular solution was increased by 3.

TABLE V.—CONCENTRATION OF AMMONIUM CHLORIDE AND QUININE HYDROCHLORIDE IN WATER AND IN 10% V/V TANGERINE

Tube Number	Normality of Ammonium Chloride in Water	Normality of Ammonium Chloride in Vehicular Solution	Normality of Quinine Hydrochloride in Water	Normality of Quinine Hydrochloride in Vehicular Solution
1	0.0044	0.0067	0.000022	0.000075
2	0.0067	0.010	0.000033	0.00011
3	0.010	0.015	0.000050	0.00017
4	0.015	0.022	0.000075	0.00025
5	0.022	0.033	0.00011	0.00038
6	0.033	0.050	0.00017	0.00056

The disguising potentials of tangerine syrup for ammonium chloride and quinine hydrochloride appear in decreasing order in Tables VI and VII, respectively.

The mean disguising potential of tangerine syrup for ammonium chloride was greater than any of the other vehicles in Table VI and appears at the top of the list. This does not necessarily mean that tangerine syrup is the best vehicle for disguising the salty taste of ammonium chloride, for it has been shown that there is no difference in the disguising ability of glycyrrhiza, raspberry, orange flowers, citric acid, and tolu balsam syrups for ammonium chloride (11). Tangerine syrup, however, is equally as efficient as any of these five.

The disguising potential of tangerine syrup for the bitter taste of quinine hydrochloride places it sixth in Table VII, which indicates that it has a mediocre value as a disguising agent for bitter tasting substances (11), although it is preferable to the last 13 vehicles listed in Table VII.

CONCLUSIONS

(1) A satisfactory method for the preparation of tangerine peel tincture by maceration of the whole peel has been devised.

(2) The precipitation method, adapted for the assay of tangerine peel tincture for its oil content, is recommended because of its greater general accuracy. The polarization method is satisfactory only when the tincture has been freshly prepared.

(3) A satisfactory method for the preparation of tangerine syrup has been devised.

TABLE VI.—AMMONIUM CHLORIDE DISGUISING EFFICACY OF TANGERINE SYRUP[a]

Vehicle	Mean Threshold in Water ± Standard Error, $m_2 \pm e_2$	Mean Threshold in Vehicle ± Standard Error, $m_1 \pm e_1$	Mean Disguising Potential ± Standard Error, $D \pm e$	Significance of Difference, $\frac{m_1 - m_2}{\sqrt{e_1^2 + e_2^2}}$
Tangerine syrup	3.33 ± 0.17	5.56 ± 0.18	2.23 ± 0.12	9.02
Glycyrrhiza syrup	3.56 ± 0.22	5.72 ± 0.29	2.16 ± 0.24	5.97
17 Vehicles in order of decreasing Mean Disguising Potentials[b]	...	...	...	...
Syrup (simple)	4.15 ± 0.26	5.15 ± 0.23	1.00 ± 0.17	2.92

[a] Table reproduced from Purdum (11) with the addition of the values for tangerine syrup.
[b] Syrup of raspberry, orange flowers, citric acid, tolu balsam, acacia, orange, aromatic eriodictyon, cherry, cocoa, N. F. V, prepared cacao, N. F. VI, thyme, wild cherry, cinnamon, althea, elixir of glycyrrhiza, compound syrup of sarsaparilla, and aromatic elixir.

TABLE VII.—QUININE HYDROCHLORIDE DISGUISING EFFICACY OF TANGERINE SYRUP[a]

Vehicle	Mean Threshold in Water ± Standard Error, $m_1 \pm e_1$	Mean Threshold in Vehicle ± Standard Error, $m_1 \pm e_1$	Mean Disguising Potential ± Standard Error, $D \pm e$	Significance of Difference, $\frac{m_1 - m_1}{\sqrt{e_1^2 + e_1^2}}$
Prepared cacao syrup (N. F. VI)	3.50 ± 0.31	8.17 ± 0.40	4.67 ± 0.26	9.18
3 Vehicles[b]	...	...	...	...
Thyme syrup	3.67 ± 0.23	7.40 ± 0.27	3.73 ± 0.25	10.60
Tangerine syrup	2.70 ± 0.13	6.13 ± 0.13	3.43 ± 0.16	19.05
Glycyrrhiza elixir	3.73 ± 0.25	6.93 ± 0.42	3.20 ± 0.34	6.52
14 Vehicles[c]	...	...	...	...
Aromatic elixir	3.38 ± 0.25	4.97 ± 0.32	1.59 ± 0.17	3.88

[a] Table reproduced from Purdum (11) except for the insertion of the values obtained for tangerine syrup.
[b] Syrup of glycyrrhiza, cocoa, N. F. V, aromatic eriodictyon.
[c] Syrup of tolu balsam, compound sarsaparilla, raspberry, cherry, cinnamon, citric acid, althea, wild cherry, simple, acacia, orange flowers, simple orange.

(4) Tests with six different filtering aids indicated that light magnesium carbonate was the filtering agent of choice in the preparation of tangerine syrup.

(5) Organoleptic tests conducted with tangerine syrup indicated that it compares favorably in taste preference with five vehicles which ranked high in taste preference tests conducted by other workers.

(6) It has been shown that tangerine syrup has a high value for disguising the salty taste of ammonium chloride and a mediocre value for disguising the bitter taste of quinine hydrochloride.

REFERENCES

(1) Hill, A. F., "Economic Botany," McGraw-Hill Book Co., Inc., New York, 1937, pp. 433–34.
(2) Atkins, C. D., Moore, E. R., and Heid, J. L., *Fruit Products J.*, **23**, 132, 152, 157(1944).
(3) Saul, Everett L., *Am. Perfumer*, **48**, 57(1946).
(4) Nelson, E. K., *ibid.*, **29**, 347(1934).
(5) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1947, p. 407.
(6) "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists," 6th ed., George Banton Publishing Co., Menosha, Wis., 1945, p. 370.
(7) Netz, C. V., THIS JOURNAL, **17**, 663(1928).
(8) Woodman, A. G., "Food Analysis," 4th ed., McGraw-Hill Book Co., Inc., New York, 1941, p. 288.
(9) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1947, p. 408.
(10) Wright, Harold N., *J. Am. Med. Assoc.*, **108**, 950 (1937).
(11) Purdum, W. Arthur, THIS JOURNAL, **31**, 298(1942).

Comparative Studies on the Toxicity of Washed Cells of *Bacillus megaterium* and Brewers' Yeast*

By ANTHONY M. AMBROSE and FLOYD DeEDS

Data are given on the continued oral ingestion of dried cells of *Bacillus megaterium* and brewers' yeast by rats for four hundred days. No toxic effects were observed in rats ingesting diets containing up to 20 per cent of *B. megaterium* or brewers' yeast as judged by growth response, hemoglobin values, organ weights, and histopathological examination of the visceral organs.

THE DRIED WASHED CELLS of *Bacillus megaterium* are used commercially as feed supplements for the purpose of supplying cobalamin (vitamin B_{12} complex). This supplement need be only a small portion of the ration since a kilogram of dry cells contains about 15 mg. of total cobalamins (B_{12} complex) (1). However, the palatable, nonhygroscopic nature of the product, together with its general resemblance to yeast in respect to ease of production, stability, amino acid composition, and vitamin content (1), makes this material of interest not only for use in animal feeds, but possibly as an additive or supplement in foods or confections for human consumption.

* Received August 18, 1953, from the Pharmacology Division, Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany 6, Calif.

The authors are indebted to J. M. Sudarsky of the Pacific Yeast Products Co., Wasco, Calif., for supplying the drum-dried cells of *Bacillus megaterium* used in this study.

Mr. Paul V. Marsh gave technical assistance with this study.

Toxicological studies have been conducted in this laboratory on the long-range effects of diets containing dried cells of *B. megaterium* as compared with diets containing brewers' yeast. Such information on dried cells of *B. megaterium* is, to the best of our knowledge, unavailable in the literature. The usefulness of the cobalamins as antianemic agents, their nutritional properties, and their role in the synthesis of biologically essential methyl groups are, however, well established (2).

EXPERIMENTAL

Drum-dried washed cells of *B. megaterium* used in these studies were produced as described in *Chemical Engineering* (3) by a modification of the process of Garibaldi, *et al.* (4). Two lots of commercially produced *B. megaterium* were used. One lot, which was fed for the first 280 days, was prepared from a strain which is on deposit in the culture collection of the Northern Regional Research Laboratory, Peoria, Ill., where it is designated as NRRL B-938R. The other lot, which was fed during the last 120 days, was prepared from a strain derived from NRRL B-1368 by selection for resistance to bacteriophage. The strains are similar with respect to cobalamin productivity and cultural characteristics.

Two experiments were conducted in which groups of weanling male albino rats weighing 44–50 Gm. each, from a strain maintained in this laboratory for many years, were used. In one experiment five groups of 5 rats each were fed on diets containing 0, 2.5, 5, 10, and 20% *B. megaterium*. In the second experiment, for comparison, three groups of 5 rats each were fed on diets containing 5, 10, and 20% brewers' yeast. The composition of the basal diet is given in Table I. Conduct of the experiments was essentially the same as previously described (5, 6). The *B. megaterium* or brewers' yeast was added to the diet by replacement of an equivalent amount of the basal diet and mixed by means of a rotary mixer. The chemical composition of the various diets is given in Table II. The body weights of individual rats on each diet and their food consumption were determined at weekly intervals during the first 250 days; thereafter, record of body weight of each rat was made at monthly intervals. At each weighing period careful observations were made as to the general appearance and well-being of all rats receiving *B. megaterium* or brewers' yeast as compared to the control rats. Hemoglobin determinations were made on two occasions. On termination of the experiments, which lasted 400 days, all surviving rats were autopsied and all organs were carefully examined for any evidence of gross changes. The liver, kidneys, and spleen from each rat were weighed, and sections of these organs and others were fixed in 10% formalin and used for histopathological examination.

TABLE I.—COMPOSITION OF BASAL DIET

Constituents	Per Cent
Corn meal (yellow degerminated)	73
Casein (ground)	10
Linseed oil meal	10
Alfalfa meal	2
Bone ash	1.5
Sodium chloride	0.5
Cod liver oil (U. S. P.)	3

TABLE II.—CHEMICAL COMPOSITION OF BACILLUS MEGATERIUM, BREWERS' YEAST, BASAL DIET AND DIETARY SUPPLEMENTS

	Per Cent				
Constituents	Ether Soluble	Ash	Crude Fiber	Moisture	Protein N × 6.25
B. megaterium (M)	1.4	8.0	9.4	7.6	43.8
Brewers' yeast (Y)	1.8	5.9	2.8	6.8	48.8
Basal diet (B)	4.6	3.5	1.9	11.4	16.9
B 80% + M 20%	4.0	4.4	3.4	10.6	22.3
B 90% + M 10%	4.2	3.9	2.6	11.0	19.6
B 95% + M 5%	4.4	3.7	2.3	11.2	18.2
B 80% + Y 20%	4.0	4.0	2.1	10.5	23.2
B 90% + Y 10%	4.3	3.7	2.0	10.9	20.1
B 95% + Y 5%	4.5	3.6	1.9	11.2	18.5

RESULTS AND DISCUSSIONS

Effect of Growth Rate on Food Consumption.—Individual growth rates of rats on the diets containing *B. megaterium* or brewers' yeast when compared at weekly intervals with the growth rate of rats on the basal diet alone were not significantly different. Table III shows the mean initial weights and gains in body weight of all rats on the various dietary supplements. It is clearly evident that all rats on the various dietary supplements for 400 days made normal gains in weight. The results of feeding *B. megaterium* or brewers' yeast on growth and weights of the liver, kidneys, and spleen and their standard errors are summarized in Table III. From these data it is evident that the values for rats on the various supplements compare favorably with those of the control group of rats.

Food consumption for each group of rats on the various dietary supplements did not vary significantly from that of the control rats on the basal diet alone. The amount of protein ingested by rats on 20% *B. megaterium* or 20% brewers' yeast was greater than that of the control rats. Protein consumption for rats on the lower dietary concentrations of *B. megaterium* or brewers' yeast, respectively, was proportionally lower than that of the rats at the 20% level. In either event rats on all supplements of *B. megaterium* or brewers' yeast received more protein than did the rats on the basal diet alone.

Pathology.—Autopsy was done on all rats on the various dietary supplements. Grossly no difference was observed between rats on *B. megaterium* or brewers' yeast and the controls. Hematoxylin-eosin stained paraffin sections of the following tissues were prepared for microscopical examination:[1]

[1] The histopathological examination and evaluation of the tissues of rats reported herein were made by Dr. Alvin J. Cox, Jr., of the Department of Pathology, Stanford University Medical School, San Francisco, Calif.

TABLE III.—SUMMARY ON THE EFFECT ON GROWTH AND ORGAN WEIGHTS OF MALE RATS ON DIETS CONTAINING EITHER B. MEGATERIUM OR BREWERS' YEAST FOR 400 DAYS

Substance in Diet		No. of Rats	Mean Body Weights (Gm. ± S. E.[a])		Mean Organ Weights (Gm. ± S. E.[a])		
Name	Wt. %		Start	Gain	Liver	Kidneys	Spleen
Control	0.0	4	46.0 ± 0.4	349.5 ± 16.2	11.65 ± 0.34	2.30 ± 0.04	0.63 ± 0.02
B. megaterium	2.5	4	47.3 ± 1.1	353.5 ± 1.4	11.81 ± 0.28	2.30 ± 0.02	0.69 ± 0.03
	5.0	5	47.0 ± 0.5	375.6 ± 12.2	11.73 ± 0.84	2.37 ± 0.05	0.88 ± 0.09
	10.0	4	46.3 ± 0.9	347.8 ± 18.7	11.68 ± 0.54	2.34 ± 0.09	0.78 ± 0.05
	20.0	4	46.5 ± 0.3	329.0 ± 4.8	11.83 ± 0.62	2.53 ± 0.19	0.61 ± 0.04
Brewers' yeast	5.0	5	47.0 ± 1.0	346.2 ± 7.6	11.08 ± 0.32	2.32 ± 0.05	0.54 ± 0.03
	10.0	4	46.5 ± 1.2	339.3 ± 15.3	11.85 ± 0.42	2.31 ± 0.11	0.71 ± 0.03
	20.0	4	45.8 ± 0.9	326.0 ± 8.5	11.34 ± 0.68	2.38 ± 0.12	0.70 ± 0.03

[a] S. E. = Standard error of the mean.

thyroid, heart, lung, liver, stomach, intestine, kidney, adrenal, spleen, pancreas, testis, and urinary bladder. In all of the organs examined, no changes were found which could be interpreted as a direct result of treatment. There were some scars in the kidneys and other evidence of mild, old pylonephritis which appeared more frequently in the rats receiving the supplements than the controls. This may have been related to the presence of bladder worms which were fairly frequent in the treated rats. All other organs were not significantly different from the controls.

Hemoglobin values obtained at 200 and at 400 days, for each group of rats, were not significantly different from those of rats on the control diet.

It has been reported by McLean and Beveridge (7) that the addition of 5, 10, or 20% of brewers' yeast to the basal diet of rats, as the sole source of protein, produced liver necrosis within 90 days. These investigators found, however, that diets containing more than 20% yeast were nonnecrogenic, suggesting the absence of a toxic factor. These same authors (7) have observed that the incidence of liver necrosis remained low or was absent on the basal diet containing 18% yeast in the presence of varying amounts of casein (0 to 7%). However, with increasing amounts of fat in the diet (5 to 70%) there was a concomitant increase in the necrogenicity of the basal diet.

Since in our studies the histopathological examination of the livers of rats on the various dietary supplements of *B. megaterium* or brewers' yeast showed no evidence of liver necrosis, it would appear that the diets were adequate in all respects and that the supplements of dried cells of *B. megaterium*, in the amounts used and under the conditions of these experiments, contained no toxic factor.

SUMMARY

1. Studies have been presented on the comparative effects of a series of diets containing dried cells of *B. megaterium* brewers' yeast, and a basal diet known to be adequate in every respect.

2. No toxic effects were observed in rats ingesting diets containing up to 20% of dried *B. megaterium* for 400 days.

3. The negative results obtained suggest that *B. megaterium* can be safely used in human and animal nutrition.

REFERENCES

(1) Garibaldi, J. A., Ijichi, K., Snell, N. J., and Lewis, J. C., *Ind. Eng. Chem.*, **45**, 838(1953).
(2) Emerson, G., and Folkers, K., *Ann. Rev. Biochem.*, **20**, 559(1951).
(3) *Anon., Chem. Eng.*, **59**, No. 11, 244(1952).
(4) Garibaldi, J. A., Ijichi, K., Lewis, J. C., and McGinnis, J., U. S. Patent 2,576,932, Dec. 4, 1951.
(5) Ambrose, A. M., THIS JOURNAL, **40**, 277(1951).
(6) Ambrose, A. M., Christensen, H. E., Robbins, D. J., and Rather, L. J., *Arch. Ind. Hyg. and Occupational Med.*, **7**, 197(1953).
(7) McLean, J. R., and Beveridge, J. M. R., *J. Nutrition*, **47**, 41(1952).

A Study of the Root System of *Hemerocallis fulva* L.*

I. Morphology and Histology

By EDWARD P. CLAUS†

In discussing colchicine-induced polyploidy in *Hemerocallis* species, including *Hemerocallis fulva* L. and various hybrid clones, Traub (1) reported that his experiments showed the effectiveness of colchicine on these plants in aqueous concentrations of 0.025 to 0.1 per cent; however, he noticed that concentrations much above 0.1 per cent usually led to the eventual death of the treated plants. He stated that his results contradicted those of Blakeslee (2) and Levan (3) who found that the colchicine-containing species (*Colchicum*) which they tested were not subject to colchicine poisoning under the conditions of their experiments. In view of his results, Traub concluded that *Hemerocallis* species should contain no colchicine, and he added that the report of Klein and Pollauf (4) who claimed to have demonstrated the presence of colchicine microchemically in *Hemerocallis fulva* L. required verification.

Preparatory to such verification studies, a number of collections of *Hemerocallis fulva* L. were made before and during the flowering season of the plant. The unusual color and external characteristics of the underground parts were observed and recorded. Since there are apparently no published reports in the literature describing the morphology and histology of the underground organs of this plant, it was deemed advantageous to undertake the present study and to follow it with microchemical and phytochemical investigations.

Hemerocallis fulva L. is a liliaceous plant that grows naturally on moist embankments and along edges of streams. It is also widely cultivated and is hybridized freely by horticulturists. The wild plant is called "common orange day lily" or merely "day lily" inasmuch as the attractive orange-colored flowers open for one day only, close by evening, and wither the following day. Collections were made in June, 1953 before the flowers had begun to appear and also in July during the height of the flowering period. The fresh material, particularly the underground parts, was compared with dried and preserved specimens gathered in 1952 and 1951. Identification was verified by O. E. Jennings, Emeritus Curator of Botany at Carnegie Museum in Pittsburg, Pa.

MORPHOLOGY

The underground portion of the plant consists of a number of types of roots arising from a structure resembling a short oblique or horizontal root-crown as seen in Fig. 1. The root system includes smooth roots for anchorage and absorption, wrinkled roots having slight swelling centrally, and other wrinkled roots terminating in definite storage organs.

In describing the genus *Hemerocallis*, Fernald (5) refers to these organs as "fleshy-fibrous roots" or "tubers" although in describing the species *H. fulva*, he uses the term "tuberous root." During the flowering period of the plant there are two types

Fig. 1.—Underground system of *H. fulva* showing the origin of the roots on the root crown; × 1/2.

* Received August 21, 1953, from the School of Pharmacy, University of Pittsburgh, Pittsburgh, Pa.
Presented to the Scientific Section, A. Ph. A., Salt Lake City meeting, August, 1953.
† Professor of Pharmacognosy, School of Pharmacy, University of Pittsburgh.
The author wishes to acknowledge the assistance of Richard M. Doughty, College of Pharmacy, University of Kentucky, in preparing the photomicrographs.

of these "tubers" or tuber-like roots: those on older roots from the preceding season which are a bright orange-yellow color, and those on roots of the current season which are almost pure white. When freshly dug and washed free of adhering soil, the intense orange-yellow color of the older roots and "tubers" is very apparent and therefore may be considered an identifying feature. When dry they darken in color, shrivel, and become soft and spongy. In contrast, the young "tubers" are grayish white when dry and remain smooth and firm. During the flowering period the roots bearing the young "tubers" are shorter than those with matured "tubers," but following the flowering season they begin to elongate.

Fig. 2.—Various types of unmodified and modified roots; × 1/3.

Mature roots attain various sizes (as shown in Fig. 2): smooth, unmodified roots often measure up to 40 cm. long and 4 mm. thick; slightly swollen roots may be 20 cm. long, the swellings being 7 mm. thick; however, the roots bearing the storage organs are seldom more than 10 cm. long, the storage organs measuring up to 25 mm. in length and up to 12 mm. in thickness. The wrinkled appearance of some of the older roots has been interpreted as a means of contraction by which the plant anchors itself more deeply in the soil. Since new roots arise each season from the upper part of the root crown above the older roots, the underground system of the plant would otherwise become exposed if it were not for root shrinkage and contraction. Thus, as the roots continually supersede each other on the upper part of the root crown, the plant appears to develop a short, oblique, or in some cases horizontal type of underground stem. However, histological structure indicates that this is not a rhizome.

From the root crown single, elongated, smooth, yellow, jointed underground structures arise which seem to function as runners but actually are thin rhizomes. They are lighter yellow in color than the roots and, since they extend from one plant to another, their size shows considerable variation. The nodes bear no stem scars and the internodes are irregular in length.

HISTOLOGY

In cross section the roots and tuber-like organs exhibit similar characteristics: a radial polyarch arrangement of from 28 to 32 alternating xylem and phloem strands surrounded by an endodermis which is slightly lignified. The rhizome has a polyarch arrangement also but the vascular bundles are fewer in number, seldom exceeding 20 in the specimens studied; the bundles are less regular in distribution and they exhibit a collateral arrangement of xylem and phloem elements with the phloem outermost. Scattered bundles are common in both the cortex and pith of the rhizome but none appear in the cortex or pith of the roots and tubers examined.

The epidermis is usually replaced by a hypoderm of two rows of lignified cells under which are two to three rows of collenchyma. In these the walls, and particularly the middle lamella, are deep yellow in

Fig. 3.—Raphide of calcium oxalate in cortical parenchyma tissue; × 440.

Fig. 4.—Individual crystals which separate from the raphides; × 440.

color. Remnants of the epidermis can be observed in young roots, and partially lignified root hairs are sometimes present. The cortex consists of approximately 28 rows of thin-walled parenchyma that are elongated radially varying from 110 to 140 μ in length and from 46 to 85 μ in width. Raphides occur irregularly in the cortical parenchyma (Fig. 3) and may be found near the collenchyma layer, in the central cortex, or near the endoderm. The raphides vary from 32 to 85 μ long and from 23 to 50 μ wide; they are contained in cells which are smaller than the surrounding parenchyma. Individual acicular crystals frequently become separated from the raphides and overlie the surrounding tissue (Fig. 4). Crystals may measure up to 58 μ in length and 2 μ in width. They do not react with alkaloidal precipitants and, since they are insoluble in water and slowly soluble in mineral acids, they are assumed to be composed of calcium oxalate. Raphides are not present in the pith.

No starch grains occur in either cortical parenchyma or in pith parenchyma; instead, these cells are frequently filled with a granular substance staining a yellowish-brown color with iodine T. S.

A characteristic endoderm surrounds the vascular bundles, the cells being elongated transradially. They are slightly lignified throughout although the radial walls usually display a greater degree of lignification. The endodermal cells vary in length from 34.5 to 96 μ and generally average about 22.3 μ wide. Directly under the endoderm is a layer of

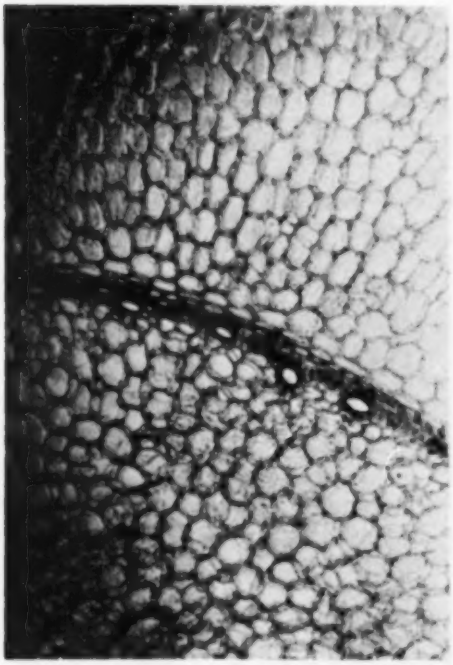


Fig. 5.—Portion of the radial polyarch arrangement of the alternating xylem and phloem strands; × 100.

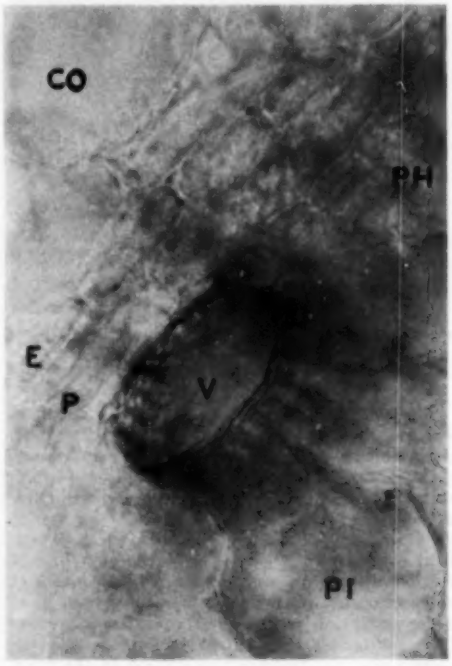


Fig. 6.—Enlarged portion of a vascular bundle. *CO*, cortical parenchyma; *E*, endoderm; *P*, pericycle; *PH*, phloem; *PI*, pith parenchyma; *V*, vessel; × 440.

pericycle cells. These are neither lignified nor fibrous, but resemble elongated, thin-walled parenchyma.

The endodermis and pericycle surround a radial polyarch arrangement of from 28 to 32 alternating xylem and phloem strands (Fig. 5). In young tubers the only lignified element is the large vessel in the xylem which averages 29.8 μ in diameter. The surrounding vessels are smaller and thick-walled but not lignified although, in time, all of the vessels become lignified. Longitudinal-tangential sections reveal that the vessels are either scalariform or pitted. No xylem fibers are present. Separating the xylem patches are phloem tissues consisting chiefly of small parenchyma and sieve tubes, as shown in Fig. 6. These blend gradually into the larger thick-walled parenchyma of the pith.

There is an absence of fibrous and sclerenchymatous tissue throughout the roots, tuber-like organs, and rhizomes.

DISCUSSION

Both old and young tuber-like organs possess the same microscopical structure, the only difference being the amount of lignification of the xylem elements. Unmodified roots as well as those leading to the tubers also show a similar histological pattern. Thus, it would be more correct botanically to replace the term "tuber" with "tuberous root."

Regarding the possible colchicine content of *Hemerocallis fulva* L., preliminary microchemical analysis of the underground organs has been inconclusive at the present writing. Further investigations of both microchemical and phytochemical procedures are planned.

SUMMARY

1. The morphology and histology of the roots and "tubers" of *Hemerocallis fulva* L. were determined.

2. Three types of roots were observed: smooth unmodified roots, wrinkled roots with central swellings, and wrinkled roots terminating in storage organs hitherto called "tubers" and "tuberous roots."

3. Older roots and "tubers" were bright orange-yellow whereas younger forms were white.

4. Raphides of calcium oxalate were prominent in the cortical parenchyma of roots and "tubers."

5. The histological structure of roots and "tubers" was identical: a radial polyarch arrangement of from 28 to 32 alternating xylem and phloem strands.

6. The term "tuber" proved to be a misnomer; the specimens examined were "tuberous roots."

7. Microchemical and phytochemical investigations will be reported later

REFERENCES

(1) Traub, Hamilton P., *Science*, **110**, 686(1949).
(2) Blakeslee, A. F., *Am. J. Botany*, **26**, 163(1939).
(3) Levan, A., *Hereditas*, **26**, 262(1940).
(4) Klein, G., and Pollauf, G., *Oesterr. Botan. Zig.*, **78**, 251(1929).
(5) Fernald, M. L., "Gray's Manual of Botany," 8th ed., American Book Co., New York, 1950, p. 433.

Book Notices

The Van Nostrand Chemist's Dictionary. Board of Editors: JURGEN M. HONIG, *et al*. D. Van Nostrand Co., Inc., New York, 1953. 761 pp. 16 x 24 cm. Price $10.

This entirely new dictionary presents information frequently needed by the chemist. The definitions included cover the several special fields of chemistry and chemical engineering. Among the more than 11,000 chemical definitions and descriptions one finds chemical substances, elements, ions, radicals, and the type compounds reflecting the latest terminology in naming compounds of silicon and phosphorus. More than four thousand chemical reactions, tests, solutions, apparatus, laws, equations, and industrial processes are defined, A unique system of cross-referencing has been employed whereby every topic is explained in basic terms and then every word important to the explanation that is further defined elsewhere in the book is printed in bold-face type to show that there is an entire article on the subject. In this way the keyword plan presents leads to further information about all related topics and assists in the correlation of information from the various fields of chemistry and related sciences. The new *Van Nostrand Chemist's Dictionary* should serve effectively as a single-volume abridged dictionary of chemical terms. The printing and binding are of good quality and the general physical appearance of the book is excellent.

Canada's Food and Drug Laws. By ROBERT E. CURRAN. Commerce Clearing House, Inc., New York, 1953. 1139 pp. 17.5 x 25.5 cm. Price $19.50.

This volume represents the fifth research book in the "Food Law Institute Series." The first four in the series dealt with the Federal Food and Drug Acts of the United States. This fifth book is an

annotated and indexed compilation of Canada's food and drug laws including cosmetic and other related laws, both national and provincial. It also includes the text and detailed comments covering an Act Respecting Food, Drugs, Cosmetics, and Therapeutic Devices, passed by the Parliament of Canada in April, 1953, but which is not expected to come into force until some time early in 1954. Thus the latest legislative record on this important subject is covered. The compilation should be of great value, not only to those concerned with the administration of Canada's food and drug laws, but also to manufacturers, distributors and retailers, and to members of the consuming public for whose benefit and protection all food and drug laws are enacted.

The Year Book of Drug Therapy. (1953–1954 Year Book Series.) HARRY BECKMAN, Editor. The Year Book Publishers, Inc., Chicago, 1953. 538 pp. 13.5 x 20 cm. Price $6.

This compact book, edited by Dr. Harry Beckman of the Marquette University Schools of Medicine and Dentistry, presents concisely the important advances in drug therapy which have occurred since the 1952 volume was published. The book is composed of an extensive series of abstracts of pertinent articles appearing in the medical literature since August, 1952. The scope of the book can best be visualized from the subject headings of the several divisions which include allergy, antibiotics and sulfonamides, cardiovascular diseases, dermatology, endocrinology, hematology, internal medicine, neuropsychiatry, obstetrics and gynecology, ophthalmology, otorhinolaryngology, pediatrics, surgery, and venereology. Thus information on recent advances of the applications of drugs in all branches of medicine is reviewed. The book is provided with an excellent subject index and an author index. It is documented by many references to the original literature and the editor is to be complimented upon his accomplishment in presenting so much information in such relatively small space.

Anleitung zur Erkennung und Pruefung der Arzneimittel des Deutschen Arzneibuches, zugleich ein Leitfaden fuer Apothekenvisitatoren. 17th ed. HANS KAISER, ed. Springer-Verlag, Berlin/Goettingen/Heidelberg, 1953. 624 pp. 17 x 24 cm. Price *DM* 32.50.

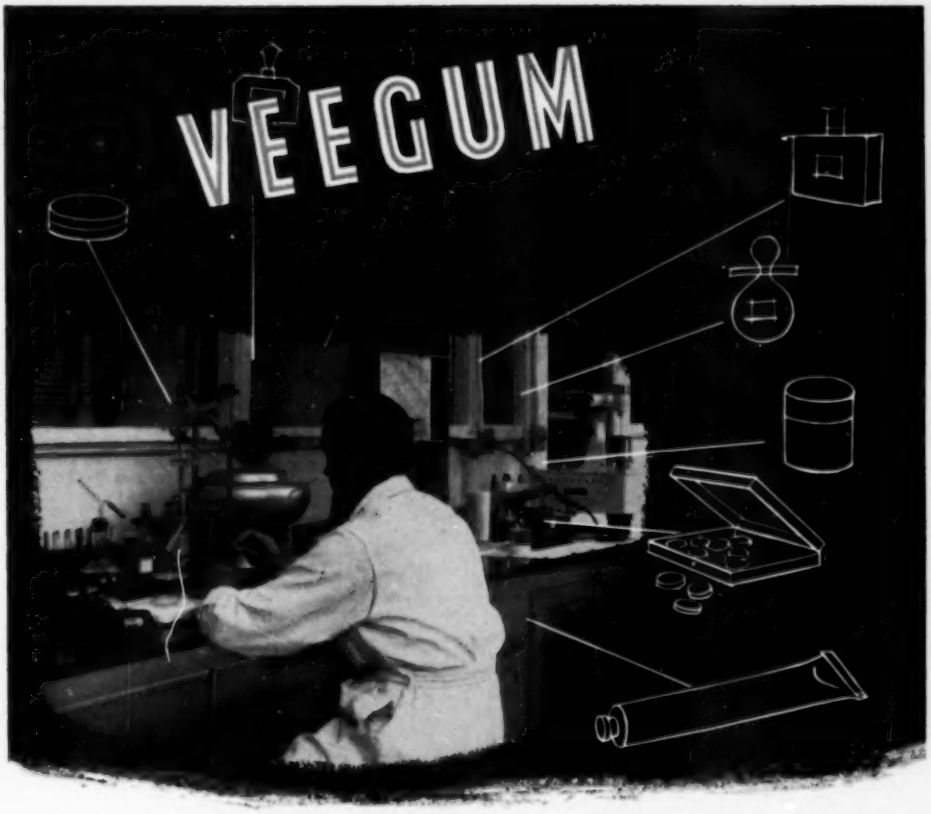
Professor Hans Kaiser has completely rewritten Ziechele and Zrieger's well-known manual on methods and standards for the remedies of the latest (6th) edition of the DAB. He has successfully incorporated the essential material from the old standard works by Herzog, Hanner, and Frerichs, and thereby has given the pharmacist an up-to-date "three-in-one" reference tool. All the information culled from those titles has been brought up to date. Kaiser's new compilation includes, therefore, besides a thorough revision of material contained in earlier editions, several additional chapters of a general nature: on the analytical methods of the *Arzneibuch*, on fats and oils, on essential oils, on determination of alkaloids, morphine, silver, mercury, on volumetric analysis, etc. At the same time, the author has wisely excluded those new methods of analysis (like refractometry or potentiometry) that require apparatus not usually available to the ordinary pharmacist. As in earlier editions, the main body of the work (some 500 pages) is constituted by an alphabetical listing of drugs, according to the Latin name as given in the *Arzneibuch*. Lists of indispensable reagents, of volumetric solutions and indicators, directions for the storage of drugs, and numerous tables enhance the value of the book. A list of laboratory equipment and supplies concludes this work which is throughout concise and to the point, in spite of its more than 600 pages. It well deserves a place on the shelves of every pharmaceutical library.

Biochemical Preparations. Vol. 3. ESMOND E. SNELL, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1953. 128 pp. 15.5 x 23.5 cm. Price $3.50.

The second volume of this series was reviewed in the October, 1952, issue of THIS JOURNAL, page 571. In that review the design and purpose of the series was discussed. The style of presentation is comparable to that in *Organic Syntheses* series and, in fact, compounds of biochemical interest which have appeared therein are listed through volume 32 in a table preceding the text. In Volume 3 the biochemical preparations for which preparative methods are given include crystalline muscle phosphorylase, ribonuclease, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), pyridoxamine phosphate, pyridoxal phosphate, protoporphyrin dimethyl ester, *d*-isocitric acid, *dl*-isocitric acid lactone, dihydroxyfumaric acid, oxalacetic acid, *l*(+) and *d*(−) lactic acids, sodium α-ketoisocaproate, *d*-glucose-6-phosphoric acid, *d*-galacturonic acid, β-2,5-dihydroxyphenyl-*dl*-alanine, *l*-isoleucine, *d*-isoleucine, *l*-alloisoleucine, and *d*-alloisoleucine, *l*- and *dl*-ornithine monohydrochlorides, *l*- and *dl*-citrulline (from ornithine), *l*-citrulline (from *l*-arginine), *l*-kynurenine, *l*- and *d*-penicillamine hydrochlorides, elaidic acid, and 2,4-dinitrofluorobenzene.

Encyclopedia of Chemical Technology. Vol. 11. (Polyols to Rutin.) RAYMOND E. KIRK and DONALD F. OTHMER, Editors. The Interscience Encyclopedia, Inc., New York, 1953. xvi + 950 pp. 20 x 27 cm. Single copy price $30; subscription price $25.

The first volume of this series appeared in 1947 and the design was described in THIS JOURNAL **37**, 130(1948). Volume 11 consists of treatises on subjects from polyols to rutin. It includes articles on subjects such as potentiometry, printing and reproducing processes, synthetic protein fibers, protein hydrolyzates, quality control quaternary ammonium compounds, natural radioactive elements, rare earth metals, reaction kinetics, natural resins, rheology, and many others of special pharmaceutical interest. When the series is completed it will represent a monumental achievement by bringing together authoritative articles on nearly every subject of chemical and pharmaceutical interest.



A Versatile Physical Conditioner For All Your Pharmaceuticals

VEEGUM enhances the physical qualities of your tablets, powders, pastes and liquids. Inorganic, non-toxic, non-irritating, VEEGUM is a completely stable suspending and emulsifying agent. Highest quality and uniformity is assured by the rigid control of the R. T. Vanderbilt Company laboratories.

IN YOUR OWN LABORATORY, you will find VEEGUM the most efficient agent for:

BINDING AND DISINTEGRATING TABLETS: An aqueous dispersion of VEEGUM, sprayed in at the granulating stage, will bind most tablets without extra operations. Its disintegrating action requires only one tenth as much VEEGUM as starch or other disintegraters.

LUBRICATING MEDICINAL POWDERS: Add the VEEGUM as a dry powder; very little is needed.

SUSPENDING HYDROPHOBIC SOLIDS: VEEGUM suspends at lower viscosity than organic gums. Thixotropic characteristics give added suspending ability.

EMULSIFYING LOTIONS and OINTMENTS: Dispersions of VEEGUM are effective emulsifiers with or without heat. Small amounts of VEEGUM stabilize many types of emulsions over a wide range of pH.

MAINTAINING PRODUCT CONSISTENCY: VEEGUM has the unique property of thickening with heat to maintain product consistency at higher than normal temperatures.

SEND TODAY FOR THE VEEGUM STORY.

R. T. VANDERBILT CO.
SPECIALTIES DEPARTMENT
230 PARK AVENUE **NEW YORK 17, N. Y.**

☐ Please send VEEGUM Bulletin B53. ☐ Please send sample of VEEGUM.
☐ Please send information on using VEEGUM for:_____

_____
(state application)

NAME_____

POSITION_____
(Please attach to or write on your company letterhead)

In the
SAME SPACE now used by
4
round bottles....
Potassium Iodide
Mallinckrodt
POTASSIUM IODIDE
U.S.P. GRANULAR
5
....you can put
Ask your wholesaler for "STORMORS"—
Mallinckrodt
PRESCRIPTION CHEMICALS
in the new STORMOR* hand tailored bottles
MALLINCKRODT CHEMICAL WORKS • ST. LOUIS • NEW YORK
*Trade Mark
OTHER ADVANTAGES ✓EASIER TO HANDLE ✓DRESSES UP THE PHARMACY ✓LABEL ALWAYS FACES FRONT ✓BETTER VISIBILITY